NASA Technical Memorandum 86654

1983–84 NASA Space Biology Accomplishments

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1983–84 NASA Space Biology Accomplishments

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Preface

The Space Biology Program currently includes forty-two research tasks. The goals, objectives, accomplishments, and future plans of each project are described in this publication as individual technical summaries. The summaries cover the period from March 1983 through March 1984.

The intent in compiling this publication is twofold. First, we wish to provide the scientific community with an annual summary of the accomplishments resulting from research pursued under the auspices of NASA's Space Biology Program. Secondly, we hope to stimulate the exchange of information and ideas among scientists working in the Program. To facilitate this exchange process, a list of publications has been included with each task summary. Specific accomplishments and conclusions drawn from the past year's research in each task have been underlined.

We would like to thank all the participants in the Space Biology Program for their cooperative response to our requests for information. We would also like to thank April Commodore Roy for her technical assistance in preparing this report.

Thora W. Halstead
June 1984

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INTRODUCTION

THE NASA SPACE BIOLOGY PROGRAM

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Introduction

One of the major features of the physical environment on the surface of Earth is the constant presence of the force of gravity. Terrestrial gravity has important biological consequences for organisms living on Earth. The phenomenon of weightlessness which is encountered on spacecraft provides an excellent biological research opportunity, both because of its uniqueness to space and because of the importance of gravity to life on Earth. Access to space provides an opportunity to manipulate gravity from its norm of one down to almost zero, effectively providing the full spectrum of gravitational research capability for the first time. This capability, combined with the stability and pervasiveness of gravity on Earth, its obvious impact on biological evolution, and its continuing effect on the morphology, physiology, and behavior of living organisms, has led the Space Biology Program to concentrate its efforts and resources on investigating the biological significance of gravity.

Program Goals

The goals of the Space Biology Program are to: use the unique characteristics of the space environment, particularly microgravity, as a tool to advance knowledge in the biological sciences; understand how gravity has shaped and affected life on Earth; and understand how the space environment affects both plant and animal species, thereby enhancing our capability to use and explore space.

Program Scope

Research in the Space Biology Program is divided into three broad areas:

1. Gravity perception. The objectives are to identify gravity receptors in organisms sensitive to gravity and determine their structure and function, and to elucidate the mechanisms by which gravitational stimuli are perceived and transmitted to a responsive site.

2. Developmental biology. The objectives are to determine the effects of gravity, and especially weightlessness, as provided by spaceflight, on the genetic integrity, cellular differentiation, reproduction, development, growth, maturation, and senescence of living systems; and to examine the evolutionary importance of gravity as a determinant of the form and function of terrestrial life.

3. Biological adaptation. This area includes the use of gravity's physiological effects to explore biological problems; and achievement of an understanding of how gravity affects and controls the physiology, morphology, and behavior of organisms, of how gravity and other environmental stimuli and stresses interact in this control, and of the biological mechanism by which living systems respond and adapt to altered gravity, particularly that of the space environment.

Research Opportunities

With the proven feasibility of the Space Shuttle, we now have a new capability of performing biological experiments in space. The opportunity has arrived to use the locker space within the Shuttle orbiter on a continuing space available basis. This will provide a valuable augmentation to the ongoing ground-based research program.

Spaceflight will provide the validation for many experimental hypotheses developed in ground-based research, while gravitational experiments on Earth will continue to hone the questions, provide the necessary baseline data, and develop spaceflight experimental protocol.

The experimental approach of the ground-based studies in the Space Biology Program is to manipulate gravity on Earth and develop weightless simulation models to: (1) develop and test gravitational hypotheses, (2) identify gravity-sensitive biological systems and interacting environmental response mechanisms, (3) analyze biological systems and mechanisms known to be gravity-sensitive, (4) analyze flight experiment data and iteratively expand ground research capability, and (5) plan and design future space experiments. In addition, research is conducted to understand how the uncontrollable biodynamic factors of the spacecraft will affect the results of the various flight experiments.

Focus of Program

The research focus of the Space Biology program is dependent upon

several dynamic factors: the requirements of NASA, the characteristics of flight experiment opportunities, the sensitivity of specific biological systems to gravity, the scientific value of the research, the state of knowledge and technology in the specific scientific areas, the interest of scientists in studying the biological questions, and the availability of funds to support the research.

Within the scope of the Space Biology Program, the current Program is focused on answering the following basic scientific questions:

1. What are the components of the gravity-sensing mechanisms of plants and animals? How do they perceive information? How is the information transmitted to evoke responses?

2. Does gravity influence fertilization and development of plants and animals, and can fertilization and development proceed normally in a near zero gravity environment? If gravity does affect fertilization and development, what are the sensitive physiological systems and how are they affected? If early development is affected by gravity, is it a result of an effect on the parent or a direct effect on the embryo itself?

3. What is the role of gravity in the formation of structural elements such as lignin, cellulose, silica, chitin, and bone calcium phosphates at the molecular level as well as at more complex organizational levels?

4. What role does gravity play in calcium-mediated physiological mechanisms and in calcium metabolism?

5. How does gravity as an environmental factor interact with other environmental factors to control the physiology, morphology, and behavior of organisms? Or, how do gravitational and other environmental stimuli interact in the control and direction of living forms? Can the action of gravity be replaced by different stimuli?

As the new opportunity to conduct biological research in space grows, an increasing amount of the research supported by this Program will contain at least some results obtained in space. The future of the Program is tied to space research.

PLANT PROJECTS

ATTEMPTS TO LOCALIZE AND IDENTIFY THE GRAVITY-SENSING DEVICE OF PLANT SEEDLINGS

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Growth Hormone Asymmetry:

Gravity induces an asymmetric distribution of the plant growth hormone, indole-3-acetic acid (IAA). The intent of this work is to find out where, and how, the hormone asymmetry originates. Such knowledge should result in the identification of the gravity-sensing device of the plant.

H. Dolk showed, 50 years ago, by means of a bioassay, that gravity induced a hormone asymmetry. To confirm this finding, an isotope dilution gas chromatographic-selected ion monitoring-mass spectrometric method was used as a definitive assay. The results both confirmed and extended Dolk's experiments, that is, the hormone was IAA and further, the IAA was asymmetrically distributed in the cortical cells of the shoot.

A further and surprising conclusion resulted from this work-that is, that the asymmetry must have originated in the vascular tissue. This is so because the preponderance of free IAA is in the vascular tissue and yet the same percentage asymmetry was obtained whether cortical tissue only or cortical plus vascular tissue was analyzed.

It is tentatively concluded that one cause of the asymmetric hormone distribution is selective and asymmetric leakage of the hormone from the vascular stele. If this is correct, it is an exciting finding since it would indicate that the gravitational stimulus can make the plant vascular tissue selectively leaky in a manner somewhat analogous to the serotonin control of capillary leakage.

Growth Hormone Catabolism:

It is now known that IAA concentration is determined by the summation of the inputs, including IAA conjugate hydrolysis, transport, and de novo synthesis--minus the outputs--including IAA catabolism and conjugate formation. Mr. Reinecke originally, and now together with H.M. Nonhebel, has discovered the major catabolic pathway for IAA in corn tissue. This is the oxidation of IAA to oxindole-3-acetic acid.

Elucidation of the further steps in the catabolism of the growth hormone IAA is planned.

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Momonoki, Y.S., Schulze, A., and Bandurski, R.S. Effect of Deseeding on the Indole-3-acetic Acid Content of Shoots and Roots of Zea mays Seedlings. Plant Physiology 72: 526-529, 1983.

Nonhebel, H.M. and Reinecke, D.M. Synthesis of High Specific Activity [5-^{3}H]- and [1-^{14}C] Oxindole-3-acetic Acid. Plant Physiology 72(1, Suppl.): 116, 1983.

Pengelly, W.L. and Bandurski, R.S. Analysis of Indole-3-acetic Acid Metabolism in Zea mays Using Deuterium Oxide as a Tracer. Plant Physiology 73: 445-449, 1983.

Reinecke, D.M. and Bandurski, R.S. Oxindole-3-acetic Acid, an Indole-3-acetic Acid Catabolite in Zea mays. Plant Physiology 71: 211-213, 1983.

IMPORTANCE OF GRAVITY FOR PLANT GROWTH AND BEHAVIOR

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Efforts during 1983 were in support of several different projects:

(1) Spacelab-1 HEFLEX Experiment. The principal objectives of the HEFLEX Experiment were accomplished. Circumnutation was observed in the μG environment. Therefore, the process does not have a mandatory requirement for a protracted G force as had been predicted for one particular model of the mechanism of circumnutation.

(2) Preliminary work continued on measurements of leaf hyponastic response to an axially directed G force. The methodology being developed will be used to test whether epinastic curvatures produced by clinostatting are attributable to a clinostat artifact or, as proposed, to a graded response to any G force imposed in line with the axis of the test plant. Results will be important for a possibly revised interpretation of data from one of the earliest plant experiments flown by NASA on Biosatellite II. [Research Task, EPIFOG]

(3) Preparations continued for a series of measurements of the liminal angle of Capsicum leaves in hypergravity. Data in the literature tell us what happened at 1 G and at μG. With the same apparatus it will be possible to extend the experiment to G levels greater than unity. (It is predicted that the system will be relatively insensitive to the G force only above 1 G). [Research Task, HYGCAP]

(4) Experimental work was completed on a study of the kinetics of damping out of circumnutational oscillations in clinostat-simulated weightlessness. A manuscript based on this work is now in preparation. By applying the currently most popular theory of mechanism of circumnutation, it was predictable that damping should have occurred within one period of oscillation. It was found that it actually required an interval equivalent to about 8 periods. That result, coupled with the more recent finding that circumnutation persisted in Spacelab μG, provided strong incentive and opportunity for a constructive modification of the gravitropic response-with-overshoot theory of the mechanism driving circumnutation. [Research Task, NULYRL]

(5) For a second year the work of a postdoctoral research fellow, Dr. D.G. Meyers, was supported. His research interests were only indirectly related to the research program but

assistance was given with the design and construction of his experimental apparatus and with the development of experimental concepts in his research area. His studies in the laboratory have led to publications in the literature of experimental limnology and to his continuing interest in further exploitation of the methodology he used.

(6) At the request of the NASA HQ Life Sciences Program Office, samples of sunflower shoots that had been grown in Spacelab μG and other samples grown at 1 G on one of the HEFLEX centrifuges during the SL-1 Mission were made available to Dr. R. Bandurski, a Program Grantee. Flight-type plant modules and soil mixture were also made available so that fully valid 1 G ground control plants could be cultured in Dr. Bandurski's laboratory. These plants will be analyzed for indoleacetic acid.

(7) Ground-based support experiments under development for the Spacelab 4 (4A?) Mission include modification of the SL-1 HEFLEX hardware for implementation of candidate experiments, GTHRES and FOTRAN. Since the SL-1 project was not funded at a level that would permit fabrication of more than one flight hardware unit, and since that unit was not available for other uses during preparation and flight of SL-1, the HEFLEX hardware unit could not be modified to make it suitable for accomplishing SL-4 objectives. Therefore, the design had to be modified and mostly new hardware had to be fabricated for GTHRES and FOTRAN.

By default, a significant part of that effort had to be supported by grant funds.

(8) For those experimenters who may need to make use of the NASA Shuttle Middeck for accommodation of biological experiments, the temperature profile to which those experiments may be exposed becomes important for planning hardware design, experimental protocol, identification of needed controls, and in many instances for the basic decision on whether it would or would not be scientifically worthwhile to fly a particular experiment in the Shuttle Middeck. Empirical data on temperatures recorded simultaneously in up to four Middeck lockers have been acquired from NASA sources and from flight packages flown in the Middeck on STS-2, STS-3, and STS-9. Those data are in the process of being collated, and, if NASA continues to acquire such information on a routine basis for each Shuttle mission, it should become possible to develop a realistic specification for the limits of an average thermal profile which poikilothermic test subjects may experience, if they are flown in the Middeck without active temperature control.

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BIOPHYSICAL BASIS OF ASYMMETRICAL PLANT GROWTH INDUCED BY GRAVITY

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This research is aimed at elucidating the mechanism by which the growth of plant stems is altered under the influence of gravity. Typically, when a plant is placed in a horizontal position, the growing region of the stem will reorient itself into a vertical position by unequal growth of the upper and lower sides of the organ (a response called gravitropism). This project examines the biophysical basis for this growth asymmetry induced by gravity. To change the growth rate of a plant cell, gravity must alter at least one of the cell properties which control water absorption and irreversible expansion of the cell wall. These properties include the cell hydraulic conductance, cell turgor pressure, the gradient in osmotic pressure across the cell plasmalemma, cell wall extensibility, and cell wall yield threshold. The immediate question is, which of these properties is altered by horizontal placement in a gravitational field.

This project began in September 1983. During the 4 mo. in 1983 in which this project was active, an apparatus was assembled for high resolution measurements of the growth and curvature along the axis of the stem. Such measurements are essential (1) for quantitating the location and magnitude of the growth response to gravity, and (2) for subsequent studies on the mechanism of the growth response. The approach taken for these measurements involves painting very fine black marks on the upper and lower sides of the stem at approximately 2-mm. intervals and photographing the marked stem at 15- to 30-min. intervals. The photographs are projected onto a digitizing tablet which is interfaced with a microcomputer, and the X/Y positions of the marks on the upper and lower surfaces of the stem are recorded by the computer. Various algorithms for calculating the distance and average curvature between any two marks on the stem were evaluated. The best technique appears to be to fit the data to a cubic spline and to estimate distance and curvature from standard formulas using the coefficients of the cubic spline. Tests have shown this apparatus and method to have the high accuracy and repeatability necessary for this work.

Initial measurements were made using pea (Pisum sativum L.) plants, but because of their rather slow and variable response to horizontal placement, more recent work has been carried out with cucumber (Cucumis sativus L.) plants. Two surprising findings, still tentative, have come from the measurements made so far. The initial growth response appears to be an acceleration of growth on the lower side of the cucumber stem. This burst in

growth is most prominent during the time from 10 to 30 min. after the start of horizontal placement and occurs along the entire length of the growing region. Little change in the upper side of the stem is seen at first. The growth rate on the lower side, however, quickly returns to the prestimulus value while at the same time the growth of the upper side rapidly declines. Thus, the curvature which develops during the first 30 min. is caused primarily by enhanced growth on the lower side. Subsequent curvature is caused by a reduced growth rate on the upper side. These observations could be of major importance for other studies which are examining the role of gradients in hormones, calcium, pH, and other substances in mediating the effect of gravity on plant growth. It is possible, for example, that the initial growth burst on the lower side might be caused by one type of gradient, while the subsequent growth suppression on the upper side might be mediated by a different factor. These findings are also significant since models of gravitropism need to account for not only the development of curvature, but also must be consistent with the detailed kinetics of how this curvature develops temporally and spatially.

Work has also begun to examine the possibility that the altered growth is due to an alteration in the osmotic pressure of the cell contents. Cucumber stems were placed horizontal and after 45 min. were bisected, separating upper and lower halves. Cell sap was expressed from these bisected halves and measured with a cryoscopic osmometer. No difference in the osmolality of the two sides was detected (mean value was 210 mOs./kg.). Measurements to be made at other points in the gravity response are in progress.

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Cosgrove, D.J. Photocontrol of Extension Growth: A Biophysical Approach. Philosophical Transactions of the Royal Society, London, Series B 303: 453, 1983.

AROMATIC BIOSYNTHESIS IN PINE TISSUES

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Higher plants synthesize more aromatic compounds than any other group of living organisms. While the biosynthetic pathways for synthesis of many of the aromatic compounds are known, the regulation of aromatic biosynthesis remains mostly obscure. The principal goal of this research is to gain an understanding of the regulation of aromatic biosynthesis in higher plants. Principal focus is on synthesis of the aromatic structural polymer, lignin, in young developing pine and mung bean seedlings. The principal area studied in 1983 concerned the effect of light on induction of lignification in pine seedlings.

When germinating pine seedlings are grown in the dark they become more elongated and less fibrous than light-grown seedlings. If etiolated pine seedlings are analyzed for lignin after a 13-day dark treatment, they contain only about 30% of the lignin of light-grown seedlings. If etiolated seedlings are then placed in light, there is a rapid increase in lignification. It is concluded that light is critical for normal lignification. In fact, low levels of lignification in etiolated seedlings appears to be a survival phenomenon which enhances the plant's ability to search for light through increased elongation.

The basic questions regarding the effect of light on lignification are:

(1) Is the light requirement principally as a source of energy for lignification?
(2) Is the light requirement principally for substrate synthesis and specific enzyme induction?
(3) How does light alter aromatic metabolism to enhance lignification?
(4) Does light activate specific genes required for lignification?

There are data pertaining to the first three questions. It appears, from experiments, that <u>light is very important as an energy source for lignification</u>. When etiolated seedlings are placed in light, the increased rate of lignification is closely correlated to light intensity. This suggests that light is not principally a trigger but an energy source for lignification. In addition, experiments in which pine seedlings were exposed to up to 60 min. of light daily did not significantly increase their rate of lignification. Again, this suggests that light is required for biosynthetic energy rather than for enzyme

induction. Still to be determined are substrate and aromatic intermediate levels in response to light in an effort to establish how light affects substrate production and intermediate pools.

One of the critical enzymes for lignin synthesis is phenylalanine ammonia lyase (PAL). PAL is known to be induced by light. Experiments on PAL activity in pine in response to light have shown a 3- to 4-fold increase in PAL activity in 2 days. Activity of peroxidase, another enzyme required for lignification, does not increase appreciably after exposure of etiolated pine seedlings to light. These results support the suggestion that PAL but not peroxidase is light induced. However, based on information discussed in the previous paragraph along with other experiments on PAL induction in pine, the increase in PAL activity may not be primarily due to light directly, but to other factors such as changes in available substrate. It appears that peroxidase activity is already sufficient to accommodate the increase in lignification that results from exposure to light. Again, there is a need to quantitate the substrate pool during light induction.

The conclusions at this point are: (1) that light is critical for lignification by supplying metabolic energy; (2) that the activity of at least one enzyme, PAL, increases when etiolated seedlings are exposed to light; and (3) that the lignin polymerizing enzyme, peroxidase, is not rate limiting in young pine seedlings.

PUBLICATIONS

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THE RELATIONSHIP OF CALCIUM AND AUXIN TRANSPORT IN ROOT GRAVITROPISM

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Auxin, indole-3-acetic acid (IAA), is a prime candidate for regulating and modulating the differential growth response of primary corn roots to gravity. IAA can promote and inhibit root elongation rapidly within a narrow concentration range. Thus, growth regulation would require only small changes in auxin flux and cellular auxin concentration which in turn could be rendered in the short lag period for initiation of gravitropism. Since auxin is transported to/through the zone of elongation toward the meristem, it may serve as a direct communication link between the zone of elongation--site of gravitropic response--and the root cap (RC)--site of gravity perception. When auxin transport is inhibited, gravitropism is also inhibited. Napthylpthalamic acid (NPA) is one such inhibitor. It inhibits gravitropism only when applied to the apical growing and dividing region of the root. Application at the basal end of the root does not influence gravitropic curvature. NPA causes upward curvature when applied to the upper surface of horizontal, 2-day-old, intact corn roots. This effect is countered by application of IAA to the opposite side. NPA affects auxin accumulation in 1-mm. slices of apical root tissue but does not affect abscissic acid (ABA, another possible mediating hormone) net uptake.

Such evidence strongly implicates IAA and its transport within the apical region in the molecular control of root gravitropism.

Calcium applied asymmetrically to the root tip will elicit curvature towards the calcium side. In addition, calcium is laterally and polarly translocated downwards at the RC of horizontal roots (Lee, Mulkey, and Evans 1983 Science 220: 1375-1376; Lee, Mulkey, and Evans 1983 Plant Physiology 73: 874-876). In light of this accumulating evidence implicating the importance of calcium in the RC in determining the direction of gravitropic curvature in roots, the relationships between IAA transport and apical calcium have been investigated. In order to establish a mechanism for communication/regulation between the elongation zone and the RC of graviresponding roots, the effects of calcium on lateral transport, acropetal/basipetal transport, and asymmetric distribution of IAA are being researched.

Corn seedlings were germinated vertically at 30° C for 2 days in light. Seedlings with straight roots 1-3 cm. long were selected. Since both removal of the RC and EDTA (a calcium chelator) applied to the RC can prevent gravitropic curvature, it has been

suggested that the source of calcium that elicits an effect on gravitropism is in the RC (Lee et al.) To test the effect of calcium and EDTA on acropetal IAA transport, the RC was gently teased off the meristem and replaced with an agar receiver block containing calcium, EDTA, or buffer alone. An apical 6-mm segment was excised from the decapped root and held vertically in a Plexiglas holder, the apical end on the receiver block. Donor blocks containing ^{3}H-IAA were applied at the base. After a 1.5-hr. transport period the receivers were collected. Calcium (1 mM) in the receiver did not affect efflux of label into the receiver. However, EDTA significantly reduced the label in the receivers whereas ABA enhanced the label content. EDTA only inhibited label effluxing from the meristem. Acropetal transport investigated in 6-mm. segments taken 3 mm. behind the RC was not effected by EDTA. The effect of EDTA at the meristem could be reversed by replacing EDTA with a calcium block. Calcium in the RC may regulate IAA translocation from the meristem to the RC.

Does asymmetrical calcium applied at the tip influence asymmetric distribution of acropetally or basipetally transported IAA? To test the former, apical 6-mm. segments were excised from decapped roots and oriented horizontally in Plexiglas holders. Radiolabeled donor blocks were applied at the basal ends. One calcium and one buffer agar block were placed in opposition on the upper and lower surfaces of the root tip. After 1.5 hr. there was no significant difference in the radioactivity of these upper and lower receiver blocks at the tip. Asymmetrical calcium at the tip did not affect acropetal transport and asymmetrical distribution of label at the tip.

Calcium did strongly affect asymmetric distribution of basipetally translocated IAA. In this case donor blocks were applied at the meristematic apex, a calcium block was placed on the upper or lower surfaces of the tip, and receivers were positioned on the upper/lower surface of the basal end of the segment. Asymmetric calcium at the tip resulted in greater accumulation of label in the basal receiver block corresponding to the calcium. Furthermore, these data reveal that calcium can be preferentially translocated across the meristem. Lee et al. found that radiolabeled calcium was only translocated polarly across the RC; when the RC was removed so was the polarity of lateral translocation. Thus, since no preferential movement of calcium exists in the meristem, then one would expect that the ratios of radiolabel found in the basal receivers (receiver on the side of calcium/receiver opposite calcium) with calcium on the upper side and with calcium on the lower side would be equal. However, these ratios are significantly different ($p=0.0066$), indicating that calcium is translocated laterally downwards across the meristem. The identity of the radiolabel collected in the receivers has been identified as IAA using high performance liquid chromatography (confirmation by mass spectroscopy is intended).

In summary, calcium has been found to affect auxin acropetal efflux from the meristem and to elicit asymmetric transport of auxin traveling basipetally from the meristem. Calcium research has suggested that graviperception results in asymmetric calcium accumulation on the lower side of the root cap and, it is proposed, may impose calcium asymmetry in the meristem. It is suggested that this calcium redistribution and asymmetry effects a reciprocal asymmetric distribution of auxin in the elongation zone by affecting basipetal transport of auxin toward the site of gravitropic response. This is the first evidence suggesting a mechanism for communication between the site of gravity perception and the site of gravity response in roots.

PUBLICATIONS

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THE ROLE OF ACID AND CALCIUM GRADIENTS IN GRAVITROPISM

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Research is directed toward understanding the influence of gravity (or lack of gravity) on plant growth--in particular the mechanism by which roots become oriented in a gravitational field and grow perpendicular to the direction of gravity. This phenomenon is called gravitropism.

Work during 1982 implicated three major factors in the control of root gravitropism: the growth hormone, auxin; hydrogen ions secreted from the roots; and calcium ions present in the root cap, the region where gravity detection occurs. The evidence implicating calcium was: (1) calcium-immobilizing agents applied to the cap caused roots to lose gravisensitivity, and (2) establishment of a calcium gradient across the root cap caused gravitropiclike curvature toward the calcium.

During 1983 calcium involvement in root gravitropism was investigated. The following questions were addressed: (1) Does gravistimulation cause redistribution of calcium in the root? If so, does this occur in the gravity-detecting portion of the root (the cap), in the gravity-responding portion of the root (the growth zone), or both? (2) Is calcium movement linked to auxin movement? (3) Can calcium gradients at the root tip influence auxin gradients in the growth zone? (4) Is calcium involved in the gravity-detection mechanism or in the growth response mechanism? (5) Is there a correlation between the effect of environmental parameters on gravitropism and on calcium physiology? These questions were studied by using radioactive calcium and auxin to measure the influence of gravity on calcium movement, the influence of the hormone on calcium movement, and the influence of calcium on the movement of the hormone.

The major findings from these studies are: (1) Gravistimulation induces polar transport of calcium toward the bottom of the root cap of gravisensitive roots. This is the most important finding from the research. It is especially significant because it relates a natural consequence of gravistimulation (development of a transorgan calcium gradient) to the observation that applied calcium gradients induce gravitropiclike curvature without gravistimulation. This suggests that a gravity-induced calcium gradient across the cap links gravity detection in the cap to the asymmetric growth that causes curvature. This link has eluded plant physiologists for decades. (2) The polar transport of calcium in caps of gravistimulated roots is linked to movement of auxin. Inhibitors of auxin transport prevent gravity-induced

polar movement of calcium. This relates this study to the work of others who have shown that polar transport of auxin in shoots depends upon calcium. The results raise the possibility of coupling calcium asymmetry to the asymmetry of auxin, the hormone most likely to regulate the gravitropic growth response. (3) The calcium antagonist, flunarizine, and the calmodulin inhibitor, chlorpromazine, interfere with the uptake of calcium by root cell protoplasts, and they prevent gravitropism. This strengthens the hypothesis that calcium movement is critical to gravitropism and raises the possibility that calmodulin is involved in the action of calcium in gravitropism. (4) Application of calcium to one side of the cap of an intact vertical root enhances the movement toward the tip of label from radioactive auxin applied to the elongation zone. This is significant because it indicates that calcium gradients in the cap may lead to auxin gradients in the elongation zone, a factor long thought to control gravitropic curvature. (5) When roots made nongraviresponsive by treatment with EDTA are gravistimulated, they show gravitropic curvature when returned to the vertical and supplied with calcium in the absence of EDTA. This shows that the role of calcium is not in the detection of gravity but in the response of the roots to a perceived gravity signal.

Toward the end of 1983, work began on the investigation of two other aspects of calcium involvement in gravitropism: (1) Examination of the changes in gravistimulated calcium movement which accompany the induction by light of gravitropic sensitivity in dark-grown roots. The results indicate that graviinduced calcium movement across root tips is light-dependent. This is further evidence that calcium movement is a primary factor in root gravitropism. (2) The redistribution of radioactive calcium within tissues of the elongation zone of gravistimulated roots was studied. Although no polar movement of radioactive calcium was seen across the elongation zone (in contrast with the root cap), there was, however, a strong asymmetry of labeled calcium within the elongation zone, with calcium accumulating along the lower side. These recent findings open up new ways of investigating the role of calcium in root gravitropism. Experiments on photomodification of graviinduced calcium movement and on calcium redistribution within tissues of the elongation zone will constitute a major part of the research effort in the coming year.

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GRAVISTIMULUS PRODUCTION IN ROOTS OF CORN

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The perception of gravity by roots occurs in a specialized region of the root known as the root cap. To understand the biochemical steps involved in translating the gravity stimulus into a growth response, the occurrence in the root cap of two specific processes has been examined: (1) protein synthesis and turnover and (2) carotenoid synthesis and turnover. Evidence has been obtained supporting the notion that both of these groups of compounds are involved in the gravity translation process. For this research, a variety of corn has been used which, if germinated and maintained in darkness, fails to exhibit any response by the primary root to gravity. Illumination of the root, specifically illumination of the root cap, with white light causes the root to respond to gravity and to bend downward.

It had been shown that inhibiting protein synthesis within the root cap prevents the root from responding to gravity when illuminated. During 1983 the involvement of protein synthesis within the cap in the processing of the gravity stimulus was examined in more detail in order to determine whether the gravity translation mechanism involves:

(1) de novo synthesis of one or more unique proteins,
(2) a general enhancement in the levels of many proteins, or
(3) an enhancement in the levels of selected proteins.

This work has shown that light markedly stimulates the levels of several groups of proteins which appear to be important in processing the gravity stimulus. However, since no de novo synthesis was shown, it is concluded that light, in part, affects gravitropic root bending by enhancing the levels of selected preexisting proteins in the root cap. Furthermore, it has been shown that in the dark these light-enhanced proteins turn over relatively rapidly, usually within a half an hour after returning the roots to the dark.

A second and now major effort has focused on carotenoid synthesis and turnover in the root cap. It has been shown that light causes a 50-60% increase in carotenoid levels in caps, compared with levels in caps maintained in continuous darkness. Not all carotenoids follow this pattern, however. Within 5 min. of illumination, a specific carotenoid, violaxanthin, decreases to a level 50% of that observed in caps maintained in continuous darkness. In caps illuminated for periods in excess of 5 min., a

60-70% reduction occurs in the original level of violaxanthin. If roots are illuminated and returned to the dark, violaxanthin levels increase to that observed in dark control root caps. This light-stimulated turnover of violaxanthin is particularly intriguing because:

(1) violaxanthin has been reported in other systems to be converted to compounds with plant growth inhibitor properties, and
(2) current hypotheses suggest that roots bend in response to gravity because of the production and asymmetric redistribution of a growth inhibitor.

The analysis of violaxanthin synthesis and turnover is continuing. In addition, examination of whether inhibitory growth substances increase in the cap concomitant with the turnover of violaxanthin has begun.

A third aspect has focused on establishing whether an association exists between carotenoids and amyloplasts, the organelles traditionally associated with gravity perception in roots. For this work, amyloplasts have been successfully isolated from root caps. To date the yields have been lower than hoped (approximately 30% intact). During 1984, improving the isolation procedure for amyloplasts will continue in order to increase the percent intact amyloplasts.

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STARCH DEPLETION AND GRAVITROPIC RESPONSE

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Considerable correlational evidence supports the contention that especially dense starch-containing amyloplasts function as statoliths for gravity perception in higher plants. The data come from three types of experiments: (1) When amyloplast-containing root caps are surgically excised, gravitropic sensitivity disappears, but sensitivity reappears when the starch-containing caps are regenerated. (2) Genetic mutants with smaller amyloplasts show decreased gravisensitivity. (3) Depletion of amyloplast starch by hormonal treatments leads to decreased gravitropic sensitivity. While this paints a generally convincing picture, correlations alone do not give proof, and more evidence is required.

It has been reported previously that gravisensitive stems of etiolated peas contain two types of amyloplasts: small unigranular amyloplasts (UGA), found extensively in cortex and pith, which do not visibly displace in a 1 x G field; and larger, multigranular amyloplasts (MGA), limited to bundle sheath parenchyma, cells that displace readily within 2-3 min. in a 1 x G field. Methods have been developed for isolating each of these in bulk, and some biochemical data have been provided for the MGA, the putative gravireceptors in this plant (Gaynor and Galston, 1983). While preparing for further molecular investigations on these MGAs another technique has been used to get more physiological evidence for the essential role of the MGA in graviperception.

It has been known for more than a century that plants exposed to near-freezing temperatures massively degrade starch to sugar. This effect has been used by several investigators to induce decreased gravitropic response and then to study recovery of full response. This technique has been used in conjunction with starch analyses to get more precise quantitative correlations between starch content and response to gravity. Individual pea plants in vials were exposed to 4° C for various periods, given a 40-min. horizontal induction in the cold, then returned to the vertical position and allowed to curve for 1 hr. at 27° C. Figure 1 shows that the gravitropic response decreases with increasing duration of cold storage, with 12 hr. of cold showing near maximal effect. Cold storage caused alpha-amylase activity to increase and amyloplast starch content to decrease, although some MGA were still visible after 6 1/2 days at 4° C. Cotylectomy coupled with cold treatment induced both a more rapid loss of gravitropic sensitivity and a virtually complete absence

of MGA. When cotylectomized, cold-treated seedlings were returned to 27° C before gravitropic induction, their response to gravity was partially restored within 2 hr. if the initial cold period had not lasted too long (Figure 2). This early recovery of gravisensitivity precedes detectable starch increase, but later recovery is correlated with more starch.

The most striking finding is that the presentation time (minimal induction time necessary to elicit a statistically significant curvature) rises as starch content declines during cold storage (Table 1). This establishes a precise quantitative correlation between these two parameters, and shows that only a ~20% reduction in amyloplastic starch is sufficient to trigger a 4-fold loss in gravitropic sensitivity. This experiment is to be repeated in an even more definitive way, that is, by analyzing MGA and UGA separately.

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Table 1. Effect of Incubation at 4°C on the Presentation Time and the Starch Content of Cotylectomized Pea Seedlings

Treatment	Presentation time	Starch content (µg starch/ mg protein)
Control 27°C	3.4 ± 1.1	118 ± 6
1.0 day at 4°C	12.3 ± 4.9	94 ± 10
3.5 days at 4°C	22.8 ± 9.6	72 ± 4
6.0 days at 4°C	60.4 ± 11.2	56 ± 9

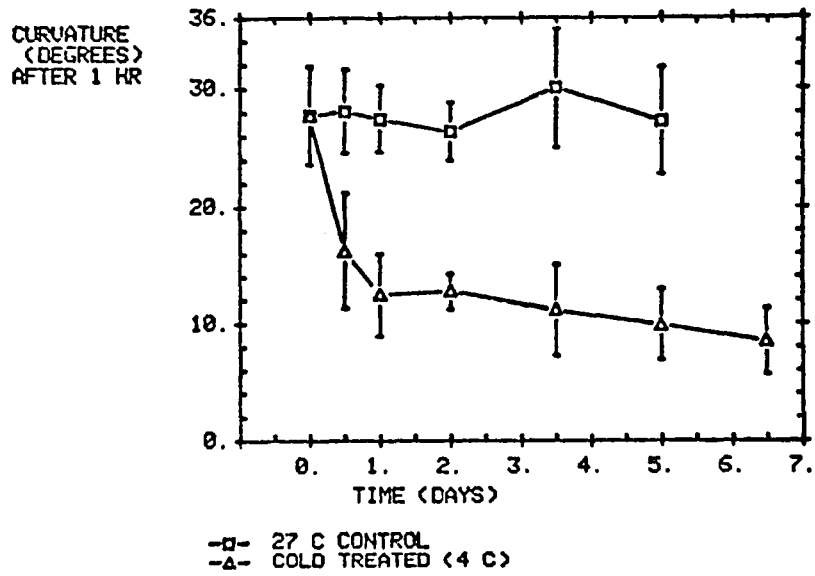

Figure 1.

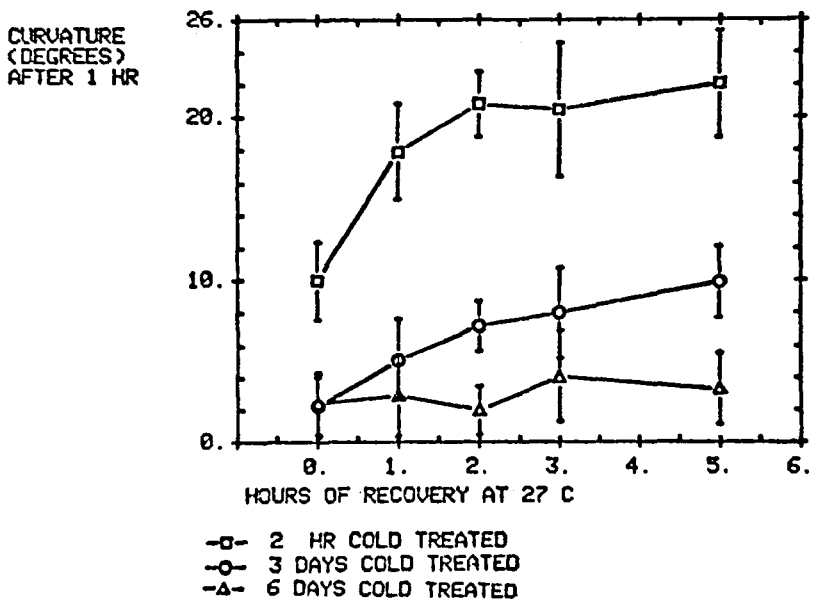

Figure 2.

THE ROLE OF GRAVITY IN REGULATION OF LEAF BLADE FORM

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The objective of this research is to understand how gravity influences the mechanisms that maintain the flat form and horizontal position of the leaf. The system studied is the nastic curvature of the primary leaves of the pinto bean (Phaseolus vulgaris L. var pinto) in response to auxin treatment, clinostat rotation, and other chemical and environmental factors. The basic question being addressed is: What causes the unequal growth that produces curvature? The related questions are: What cells are involved? Why do some cells respond and not others? How do they respond--elongation or inhibition? What are the roles of auxin and ethylene in the cellular response? How do cellular responses relate to the responses of the whole leaf in determining blade form and position?

The following methods have been used:

(1) Whole leaf responses to various treatments have been quantitated by bioassay techniques developed by this laboratory to monitor curvatures of blade, petiole, and pulvinus; (2) standard gas chromatography has been used to determine the time course, location, and stimuli for ethylene production by the leaf; (3) cytological studies, using scanning electron microscopy, epidermal films, and histological sections, have helped identify and localize cellular responses; and (4) transport of labeled indole-3-acetic acid (IAA) through leaves has been studied by standard donor-receiver techniques and quantitated with a liquid scintillation counter.

Comparison of hyponastic with epinastic leaves has revealed that both the upper and lower epidermal cells can respond to auxin treatment by differential elongation, producing curvature. However, the upper epidermis does not respond to auxin treatment unless an inhibitory agent, such as an auxin transport inhibitor (TIBA, NPA), ethylene, or clinostat rotation, affects the distribution of auxin in the leaf. Auxin distribution and the differential sensitivity of upper and lower epidermal cells determine whether the response will be epinastic or hyponastic.

Studies during 1983 have also shown that it is the interaction of blade, pulvinus, and petiole, rather than the response of any one part of the leaf, that constitutes the significant response to orientation to gravity. It has been demonstrated that leaf blade response is auxin regulated, leaf angle response is ethylene requiring, and that the two responses are linked by the

relationship between auxin and ethylene synthesis in the blade.

The important observation that dorsally applied ^{14}C-IAA accumulates in ventral receivers when a blade tissue section is in a normal orientation to gravity, but that it does not so accumulate when the blade is inverted, is the first demonstration of dorsiventral transport through the blade. Earlier studies with auxin transport inhibitors, inversion and clinostat treatments, and other incubation conditions had anticipated this observation.

Other factors that affect nastic curvature were also investigated. It was found that low pH buffer did not promote hyponasty in statistically significant amounts, nor did sodium vanadate, an inhibitor of ATP-driven proton secretion, produce significant epinasty. However, when applied to the lower surface of the leaf opposite the IAA upper, it did inhibit hyponasty. Also, it had been noted earlier that dark treatment promotes hyponasty and bright light inhibits it. Responses to red, far red, blue, green, and white light are now being examined. Preliminary studies show blue light inhibits hyponasty and far red promotes it.

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EFFECTS OF GRAVITY AND LIGHT ON GROWTH, DEVELOPMENT, AND FLOWERING IN PLANTS: II. MULTIPLE GENERATIONS ON CLINOSTATS

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The effect of a long-term microgravity environment on the life cycle of plants is poorly understood. Whether higher plants have evolved to a stage where removal or reduction of gravity is detrimental to the plant life cycle and thus fatal to the plant species is an unanswered question. In space, plants have been successfully grown through the various stages of their life cycle. Attempts to grow plants in space as a continuous integral process from seed to seed through one generation have not been successful until recently.

Culture of plants through multiple generations has not been accomplished in space or in ground-based simulated weightlessness studies. The effect of long-term simulated weightlessness is being investigated by growing three consecutive generations of plants continuously on clinostats using the cruciferous plant Arabidopsis thaliana (L.) Heynh.

Three generations of Arabidopsis have been grown on clinostats. Two generations (F_1 and F_2) were successfully grown from seed-to-seed on clinostats. The third (F_3) generation is now being tested. In analyzing leaf, stem, and seed pod development of the F_1 and F_2 generations, a significant delay in the formation and development of seed pods was found in both generations of clinostatted plants, compared with vertically rotated and stationary upright controls. Attention will be focused on the delay response in the F1 and F_2 generations to see if the delay response appears again in the F_3 generation. Soviet workers have recently reported a similar delay in Arabidopsis grown in space through one generation. This delay found in plants grown both in space and on clinostats may indicate that gravity is a requirement during the reproductive process of Arabidopsis.

Seeds representing the F_1 generation were tested for viability. The crop was divided into those seeds from dry mature pods and those from pods still green at harvest. The germination rate was significantly higher for seeds from dry pods (95.9 $\pm$ 0.9%) than those from green pods (83.6 $\pm$ 2.5%). Abnormal seedlings were significantly lower in the dry pod population (0.37 $\pm$ 0.26%) than in the green pod population (1.69 $\pm$ 0.21%). These results will be compared later with seeds from the F_3 generation grown on clinostats.

The effect of closed cultures on the growth and development of

Arabidopsis was studied. The closed-culture environment represents typical cultural conditions found in plant experiments conducted in space. Closed cultures sealed with vinylidene polymer film had little or no gas exchange with the ambient atmosphere. Cultures plugged with polyurethane foam and having ample gas exchange were used as controls. Closed-culture plants, compared with controls, produced a larger number of leaves, stems, and flowers but did not form seed pods. It was not known whether low CO_2 concentration or accumulation of metabolic products such as ethylene or excess moisture were singly or in combination responsible for the developmental and morphological differences. Thus, a CO_2 analysis of the in vitro head gas of closed Arabidopsis cultures was conducted. Instead of the expected concentrations of less than 300 ppm of CO_2, concentrations of around 146,000 ppm and a concomitant decrease of O_2 concentrations were found in closed 8-wk.-old cultures. These results are compatible with those from a 1-wk. STS-3 flight where unexpectedly high levels of CO_2 were detected (50,000 ppm). In the experiments reported here, high CO_2 levels appeared to coincide with bolting and flowering. A daily rhythm in CO_2 concentration was also found. Preliminary tests for bacterial contamination have proven negative. Further studies are necessary to identify the source of the CO_2 in closed cultures.

Growth and morphological data have been obtained for Arabidopsis plants grown for two generations on clinostats. A significant delay in seed pod formation was found in plants subjected to simulated microgravity. Since no information is available on the effect of continuous true weightlessness on the growth, flowering, and seed production in sequential generations of plants, plans are being made to carry out studies in space using Arabidopsis thaliana (L.) Heynh.

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A STUDY OF THE MECHANISM OF THIGMOMORPHOGENESIS IN PLANTS, WITH SPECIAL REFERENCE TO THE ROLE OF ETHYLENE AND ITS SIGNIFICANCE TO RESEARCH WITH PLANTS IN SPACE

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The objective of this research project is to gain a thorough understanding of the response of plants to physical force. Emphasis is placed on those forces that result in mechanical stress and strain on the plant tissues. The most common forms of such forces are wind blowing through plants, the resistance of soil particles to emerging seedlings, animals and other plants rubbing against the plants, and the force of gravity, omnipresent on the surface of the Earth, pulling on plant tissues. The adaptive response by plants to such mechanical perturbations is known as thigmomorphogenesis. Gravitropism, the directional response of plants to changes in gravitational orientation, has many characteristics in common with thigmomorphogenesis and may be a result, in part, of a common mechanism.

During 1983, research has primarily focused on the role of ethylene in thigmomorphogenesis and its relationship with the rapid deposition of the beta 1-3, glucan, callose, and the production of elicitors. Also, since any handling constitutes a mechanical perturbation of the plants, development of nonintrusive methods of examination using computer-assisted video image analysis have continued.

Evidence now quite clearly indicates a role for ethylene in the response of plants to mechanical force. As a result of exposure to mechanical perturbations, ethylene is produced from increased synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC). ACC production increases within 20 min. of mechanical perturbation and is converted to ethylene which becomes detectable about 45 min. after perturbation and peaks at 4-5 hr. The ethylene produced triggers a wide variety of changes in growth and development of the plant, generally resulting in a plant of shorter final height and increased girth.

Analysis of these changes in growth pattern has been facilitated by a computer-assisted image analyzer which can quantify callose deposition and measure changes in cell size and shape, as well as whole plant dimensions. Examination of pine seedlings has indicated an increase in bark and resin production, following mechanical perturbation, as well as decreased shoot and needle growth. In bean and tomato plants, mechanical perturbation not only reduces elongation but inhibits the formation of a hollow

stem (pithiness development). This appears to reduce the effects of drought stress.

To better understand these types of developments, an examination of subcellular structure is continuing. Of particular interest has been the observation of numerous changes in the membrane lipid and protein complement of the cells. Mechanical perturbation results in reduction of the unsaturated to saturated ratio of the fatty acids. Phosphatidyl choline levels decrease and phosphatidyl ethanolamine levels increase. Membrane-associated protein levels increase as does the activity of Golgi-associated IDPase, but cytochrome C reductase levels decline.

Little is yet known about the events leading to the increased production of ethylene in mechanically stressed plants. However, at least two early observable events, preceding or concurrent with the production of ethylene, appear closely tied to ethylene production. The deposition of callose in the cell walls occurs almost immediately following any stress. This deposition peaks 5-6 hr. later and then callose levels in the tissue slowly decline over the next 12-24 hr. An inhibitor of protein glycosylation (DDG) has been shown to block callose deposition as well as ethylene synthesis and thigmomorphogenesis. This compound has also been shown to block gravitropism in etiolated corn and pea shoots. Furthermore, examination of the activity of beta 1-3 glucanase, the callose degrading enzyme, indicates a rise in this enzyme's activity following mechanical perturbation, which may account for the decline in callose levels commencing about 6 hr. after mechanical perturbation.

Elicitors are relatively small molecules of varied structure that are released by plants in response to some biotic or abiotic stress. They are called elicitors because they induce the production of "stress metabolites." One of these metabolites is ethylene. Elicitors have been shown to be produced by bean plants in response to mechanical perturbation. Furthermore, an elicitor extracted from bean plant cell wall material has been further shown to induce ethylene production in nonperturbed plants and result in an increased thickening of the stems. Known elicitors, from other systems, have also been shown to promote ethylene production in beans and increased radial growth.

In experiments begun at the end of 1983, attempts were made to treat plants with beta 1-3 glucans to see if they will induce ethylene production. So far, indications are that beta 1-3 glucans, when applied exogenously, do induce ethylene production.

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GRAVITY PERCEPTION, TRANSDUCTION, AND CELLULAR RESPONSE IN GRAVISTIMULATED SHOOTS OF CEREAL GRASSES

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The specific objectives in this project are to shed light on how gravity is perceived in cereal grass shoot pulvini, learn how and when hormone asymmetry is established during transduction in gravistimulated pulvini, and what the physiological and biochemical bases are for the asymmetric growth response that occurs during upward bending of gravistimulated cereal grass pulvini.

In an effort to learn how gravity is perceived in grass shoot leaf-sheath pulvini, the kinetics for fall of the presumed graviperceptive organelles, the starch statoliths, were determined. In oats and barley, they start to descend in gravistimulated shoots within 15 sec., and essentially all (ca. 50 per cell) have reached the bottoms of the statenchyma cells (located just inside the ring of vascular bundles) within 2 min. This is well before the pulvini start to bend upwards, namely, 15-20 min. after first being gravistimulated.

In order to find out whether starch statoliths in the pulvini are, in fact, the graviperceptive organelles, pulvini were treated with alpha amylase (from hog pancreas, which hydrolyzes only alpha 1,4-linked glucan = starch). After a 24-hr. treatment on a slow shaker in the dark, the amyloplasts were observed, microscopically, to be devoid of all starch (based on I_2KI staining). Furthermore, such pulvini did not show upward bending when placed horizontally. Such alpha amylase-treated pulvini were then placed in 0.1 M sucrose for a second 24-hr. period, in an upright position. They were found not only to have resynthesized starch in the amyloplasts, but also to have regained the ability to bend upwards when gravistimulated. It is thus seen that <u>starch statoliths in the leaf-sheath pulvini are indeed the graviperceptive organelles, as they are in root caps</u>.

Further progress was made in learning which hormones are asymmetrically distributed in gravistimulated pulvini of both oats and maize. They include three types of hormones: gibberellins (GA_3, GA_4, and GA_7 in oats; GA_1, GA_4, GA_8, and GA_{20} in maize); auxin[3] (indole-3-acetic acid, or IAA);[4] and ethylene. The ethylene asymmetry in oat leaf-sheath pulvini is only first seen 6 hr. after shoots are gravistimulated, almost 5.5 hr. after shoots first start to bend upwards. So <u>the production of ethylene is the result of gravistimulation</u>, probably resulting

from either the stress imposed by gravitropic bending, or more likely, the asymmetry in IAA which occurs in gravistimulated pulvini. The latter is believed to be true because in gravistimulated maize seedlings, auxin asymmetry (top vs. bottom half, with significantly more in the bottom half), as expressed by amounts of free IAA present, is seen as early as 15 min. after seedlings are gravistimulated. Tissue fractions of pulvini of oats were prepared to determine the precise time course for establishment of asymmetry of both native GAs and free IAA and their respective conjugates from the time that bending first begins until it ceases. It is already known that the asymmetry in free GAs and free IAA is well established after 24 hr. of bending, when the shoots have bent upwards to about 30^{o} (and that GA conjugates show the reverse ratio as the free GAs--more on the tops for the conjugates and more on the bottoms for the free GAs). It is now essential to determine when the asymmetry is first established for both hormones in relation to the time course of upward bending--a major project started in 1983 and due to be completed in the summer of 1984.

In connection with elucidating the biochemical and physiological bases for the asymmetric growth response in gravistimulated grass shoot pulvini, extensive polyacrylamide gel electrophoresis and isoelectric focusing were done to analyze protein changes, especially since it is already known that protein synthesis is essential for the bending response to occur. As early as 2 hr. after shoots are first gravistimulated, changes in protein occur in tops vs. bottoms of horizontally placed pulvini, as compared with "left" and "right" halves of upright pulvini. A significant increase is seen in amounts of five specific acidic proteins in the bottom halves, as compared with the top halves. They are currently being identified. One may be a cellulase; another may be invertase. Wall-loosening enzymes are likely candidates, as are enzymes involved in cell wall synthesis (the latter process, like protein synthesis, is essential for gravitropic bending to occur). Finally, methods have been devised for analyzing the activities of key wall-loosening enzymes during the entire time course of upwards bending in gravistimulated oat shoot pulvini.

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PLANT CELLS, EMBRYOS, AND DEVELOPMENT IN SPACE

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The ultimate objective of the overall research plan has been to ascertain whether flowering plants can carry out their full growth, development, and reproduction in a near-zero or hypogravity environment. Highly responsive experimental systems are being developed at different developmental or organizational levels--that is; (1) free protoplasts from higher plant cells which can deposit new cellulose walls, divide, multiply, embark upon organized development, and ultimately give rise to organized plantlets; (2) free somatic cells which by division under aseptic and heterotrophic conditions may express morphogenetic competence and form somatic embryos which can, in turn, develop into plantlets; and (3) at the level of plants as they develop from seeds or predetermined growing points.

The daylily (Hemerocallis) monocotyledonous system which has been developed provides an excellent model for studying development in space because plantlets can now be generated equally efficiently from aseptically cultured cells, protoplasts, and tissues. The chromosomes are relatively large and few in the diploid state (n=11); these have been studied in detail each step along the way to full development. Efforts have continuously been made to improve precision in these procedures. But it has not been possible to carry out chromosome analysis on materials that have not been treated with cytostatic agents such as colchicine, vinblastine, or 1-bromonaphthalene. These cytostatic agents can easily be used in an on-Earth setting but it is impossible to envision their use in space without a sophisticated piece of hardware being developed. This hardware would have to expose materials (root tips, for example) to a predetermined level of the cytostatic agent for a specific period of time at a relatively low temperature (say 10° C). This procedure would be followed by a thorough rinsing and then another exposure to a fixative (generally comprised of ethanol and hydrochloric acid) at a still different temperature and environment. All this, in turn, requires still other procedures. So while it may sound easy to say materials can or will be fixed in space, the connotation of "fixation" varies with the task to be achieved. Straightforward fixation, as performed for light or electron microscopy, may be difficult enough in itself to achieve in a space environment, but for karyological or chromosomal investigations, inflight fixation presents problems at a still greater level of difficulty.

The utilization of cold treatment as a cytostatic agent has been investigated. This would permit vastly greater degrees of flexibility for studying cytological events in the space environment. Materials could be chilled prior to flight to suppress or reduce their cell division activity. Once in orbit, ambient or physiologically permissive temperatures could be set in motion, and, once again, at critical stages in development, plant materials could be chilled or exposed to cold to arrest the processes. The material once chilled to a sufficient level could be maintained quiescent until recovery when the so-called "fixation" stage could be carried out. Numerous tests carried out during 1983 demonstrate that this is a viable approach, and efforts have been made to define precisely the level and length of cold exposure required to arrest oats, daylily, and sunflower in metaphase, and to evaluate the quality of the preparations in terms of acceptability for karyotype preparation and measurement. Some 72 hr. of cold (1°C) is needed to fully bring about metaphase arrest in oats. In terms of potential Shuttle investigations, this means that an experiment could, for all practical purposes, be terminated in space well before recovery; similarly, just before deorbit, materials could be exposed to cold. The potential means whereby the effects of reentry, etc., vs. actual microgravity can be distinguished seem feasible. The daylily and sunflower similarly appear amenable to cold as well.

It is anticipated that the successful use of cold can be extended equally well to cells and protoplasts. Preliminary experiments indicate that this will be possible.

Another feature of the work during 1983 has been the rigorous establishment of an explantation procedure for daylily from meristems of karyotyped plants which permits exact chronological scheduling of fresh suspension cultures for study without the intervention of a phase of growth on semisolid media. Explants are directly placed in liquid. The precision with which the final desired product can now be achieved is thus shortened; this eliminates any undesired changes in the cell types.

It is hoped, further, that knowledge of the use of cold will permit clarification of the kind of instrument that might best be designed for cytological studies.

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THE AMYLOPLAST AS A GRAVITY-SENSING DEVICE IN PLANTS

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During 1983, the primary focus has been determining the dynamics of the gravity-induced reorientation of amyloplasts in the statocytes of corn seedlings. While there is a large body of evidence that the movement of amyloplasts plays a central role in gravity sensing, the transduction mechanism for converting amyloplast movement into a physiological gradient has not been identified. Knowledge of the parameters of the dynamics of amyloplast movement, especially during the presentation time, is essential in order to develop relevant hypotheses about the nature of the transduction mechanisms.

In corn coleoptiles, the presentation time (the minimum time that a seedling needs to be horizontal to induce subsequent gravitropic curvature) has been determined using a log plot of the gravistimulation period against curvature (measured 90 min. after the start of gravistimulation). Because the amount of curvature increases roughly logarithmically with increased gravistimulation, this type of plot approximates a straight line. The intercept with the abscissa, which will be the minimal time for a gravitropic response, yields a value of 40 sec. Thus, the dynamics of amyloplast movement within this 40-sec. period will be of particular interest.

Utilizing coleoptiles that were fixed after various periods of gravistimulation and then sectioned, a similar plot for amyloplast redistribution in the cell has been made. The intercept of the log of gravistimulation time against the number of amyloplasts close to the new lower wall gain yields a straight line, and the intercept of this line with the abscissa yields a value of 11 sec. for the cells on the upper side of the coleoptile and 15 sec. for those on the lower side. Thus, the commencement of amyloplast arrival to the new lower wall during gravistimulation occurs in less than half of the presentation time. Furthermore, up to 21% of the total sedimentation occurred within the first 30 sec., and this amount of sedimentation was statistically significant when compared with zero time (vertical) control plants.

Observation of amyloplast movement was also made in living statocytes using a horizontal microscope equipped with Nomarski optics and a video camera and recorder. These observations confirmed that rapid sedimentation occurs in living cells as well.

Collectively, these measurements of sedimentation dynamics are

consistent with the classical statolith hypothesis that the transduction of amyloplast movement into a physiological gradient may occur near the plasmalemma of the new lower wall.

Living cells were also examined by video microscopy to observe the process as well as the kinetics of amyloplast sedimentation. Sedimentation is decidely not a simple case of inert spheres falling in a Newtonian fashion in response to gravity. Amyloplasts move in a heterogeneous fashion and cytoplasmic streaming is clearly involved in producing this variability. Amyloplasts move fastest when falling through cytoplasmic streams flowing in a downward direction. Conversely, sedimentation may be deferred for a substantial period of time if the amyloplast faces an upward stream. The range of the rates of amyloplast movement (0-210 μm./min.) was found to be much larger than had been calculated by previous workers from fixed material (0.3-1.2 μm./min.). This high range in rates would not be detected in fixed tissue where average rates are determined for whole populations of amyloplasts rather than by direct calculation of rates for individual amyloplasts in living cells.

Streaming affects not only the rates of sedimentation, but also the pathway that individual amyloplasts follow during the gravity response. It is common for amyloplasts to be swept along by the streaming cytoplasm into a lateral or even an upward direction. These perambulations of amyloplasts bring them into contact with numerous other organelles, especially the vacuole. Interaction of moving amyloplasts with other cytological structures such as endoplasmic reticulum or possibly microtubules or microfilaments has not yet been observed in living cells.

Cytochalasin B, which halts cytoplasmic streaming, has been employed to explore the relationship between cytoplasmic streaming and amyloplast movement. Methods have been developed for introducing this poison into living sections while they are under observation with the video microscope. When the poison suspends streaming, the rate of amyloplast sedimentation is somewhat reduced. These results suggest that cytoplasmic streaming may play a role as an amplifier of the amyloplast component of gravity sensing in the corn coleoptile.

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MECHANICAL REGULATION OF PLANT GROWTH AND DEVELOPMENT

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On Earth, gravity is the predominant physical force governing plant orientation, and contributes to the pattern and amount of mechanical tissue deposition within the plant as well. Seismic (shaking) and vibric (vibrational) forces resulting from wind action alter patterns of mechanical tissue formation, and certainly limit plant growth in the outdoor environment. Furthermore, traumatic (wounding), haptic (contact), and thigmic (rubbing) stimuli caused by certain types of precipitation, animal movements, etc., certainly contribute to the overall effects of mechanical stress on plant growth and development. In space, the static force of gravity is lacking, but dynamic physical forces such as shaking and vibration may assume an even more important role in altering or directing the growth of plants within an orbiting spacecraft, compared with on the ground. On the one hand, there is concern that spacecraft vibration, particularly if vectorial, may mimic the force of gravity and confound attempts to determine effects of true weightlessness on plant development; on the other hand, a little physical stimulation may benefit plants by stimulating the development of mechanical support tissue that might be needed for normal development. The net effects of mechanical stresses on plants growing under otherwise microgravity conditions awaits the results of experiments to be performed under real spaceflight conditions.

Soybean (Glycine max (L.) Merr. cv. Wells II) plants grown and shaken in a greenhouse or growth chamber on the ground develop shorter and thinner internodes, smaller leaves, and roots and shoots with less fresh and dry weight than do undisturbed control plants.

Seismic stress is applied by shaking soybean plants at 220-240 rpm on a gyratory platform shaker. Five minutes of gyratory shaking applied three times daily retarded dry weight gain of vegetative soybean plants by 25% and leaf area growth by 27% over a 12-day treatment period in a growth chamber. Growth dynamics analysis revealed that a concomitant decrease in relative growth rate (RGR) was due entirely to a reduction in net assimilation rate (NAR) in the growth chamber. An inhibition of NAR implies a decline in photosynthetic efficiency caused by seismic stress.

Five minutes of gyratory shaking caused a temporary decrease in whole plant transpiration rate of 15-17% that lasted for at least 90 min., as well as a 39% increase in leaf water potential 30

min. after treatment. Both effects are consistent with a transitory, stress-induced reduction in stomatal aperture. Photosynthetic rate (Pn) fell 10% during the 5-min. shake period, and continued to decline to 16% below controls 15 min. after treatment ended. Pn of shaken plants still averaged 12% less than that of controls 50 min. after treatment. Seismic stress also caused an immediate leaf droop of 31^{o} from the equilibrium angle at the secondary pulvini immediately after shaking, a response from which the plants did not fully recover for as much as 2 hr. Leaflets of soybean plants never having been shaken previously recovered to their equilibrium position more slowly than did those of previously shaken plants. The extent to which the seismic stress-induced decline in RGR, NAR, and Pn are due to leaf reorientation vs. leaf resistance is under investigation.

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TESTING A HYPOTHESIS: HOW THE PEA STEM SENSES GRAVITY, FRICTION, AND FLEXURE

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A new model for reception by plant stems of gravity and of the related stimuli of friction and flexure is being tested. The model specifies (1) that all these stimuli increase the leakage of Ca^{2+} across the plasmalemma and that in consequence certain proteins are phosphorylated; (2) that gravitropic phosphorylation activates a protein which transports auxin out of the lower sides of the cells, leading to a lateral migration of auxin across the stem and to differential stem elongation; and (3) that reception of friction and flexure involves phosphorylation of alpha- and beta-tubulins, which polymerize to form a "DeLorenzo polymer," ultimately causing synthesis of ethylene and consequent thickening and strengthening of the stem.

The first phase of work, now completed, was testing the electrophysiological predictions of the model. A postulated consequence of the phosphorylation of alpha- and beta-tubulin is exocytosis of vesicles containing material which, upon release, depolarizes cell membranes. Transients, ranging in size up to 600 μV. and possessing risetimes (10-90%) of approximately 200 msec. are now demonstrated to be elicited by friction and by AG2-187, an ionophore for Ca^{2+}, as well as by indoleacetic acid. According to the model, gravitropic Ca^{2+} leakage occurs through more specialized channels and the ion binds immediately to the proteins effecting phosphorylation; nevertheless a little leakage to the cytosol is expected. A 1.6-fold increase in voltage transients is now documented. This result, which is statistically reliable, is considered to be a striking success for the model.

Current experimentation addresses phosphorylation occurring during gravitropism and flexural stimulation. After pretreating etiolated pea stems with ^{32}Pi, stimulating, freezing in liquid nitrogen, extracting with appropriate buffers and inhibitors of proteolysis and dephosphorylation, running SDS polyacrylamide gels, and exposing the gels to X-ray film, there have been strong hints that phosphorylation of certain proteins increases. However, by letting the extracts sit for a few minutes before preparing samples for the gels, it could be demonstrated that phosphatase activity was not adequately inhibited. Thus, it is not known whether the increase in radioactivity that has been seen in certain protein bands is a product of preferential phosphorylation or preferential dephosphorylation. Consultation with others working on phosphorylation in plants has revealed

that problems with phosphatases are quite general. Efforts are being extended to find suitable phosphatase inhibitors, but the experiments with other plants are also being repeated in the hope of finding material with less vigorous phosphatase activity. It is felt that these efforts are valuable, for it seems evident that phosphorylation is an important regulatory mechanism in plants, and the technical difficulties must be overcome.

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THE MECHANISM OF PLANT SHOOT GRAVITROPISM

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Research is directed toward understanding how gravity influences plant growth. More specifically, interest is focused on how plant shoot systems detect a gravitational field and transduce this information into an oriented pattern of growth. During 1983, research was focused in two areas: (1) What is the role of the plant growth hormone auxin in shoot gravitropism? and (2) What role, if any, does calcium play in the ability of plants to detect and respond to a gravitation stimulus?

In order to assess whether auxin plays a key role in shoot gravitropism, an attempt was made to kinetically dissect three seemingly interwoven phenomena: asymmetric growth, auxin redistribution, and asymmetric proton efflux. Using light-grown sunflower seedlings, it was established that gravitropic curvature is initiated in 25-30 min. This curvature response results from enhanced growth on the lower surface and reduced growth on the upper surface, relative to vertical controls. In gravitropically stimulated hypocotyls, auxin redistribution (followed with ^{3}H-IAA) is detectable in 20-25 min. This redistribution occurs simultaneously and uniformly along the growing axis.

The above data suggest but do not prove that auxin is the gravitropism-mediating hormone. That is, one might argue that asymmetric growth causes auxin redistribution rather than vice versa, since the kinetics of both phenomena are quite similar. In an attempt to sort out cause from effect, auxin redistribution was followed in hypocotyls submerged in neutral buffers of high and low molarity. (High molarity buffers prevent asymmetric acid efflux and gravicurvature, while shoots in low molarity buffers respond normally.) It was found that auxin redistribution was kinetically identical in both cases. These results, therefore, demonstrate that auxin redistribution can occur in the absence of asymmetric growth and asymmetric acid efflux. It appears, then, that hormone redistribution is directly initiated by gravity perception mechanisms and not by a secondary event. Using a variety of other treatments (e.g., osmotic shock, inhibitors of auxin transport, etc.), it was confirmed that auxin redistribution could occur under conditions that prevented curvature. Further, no conditions under which the reverse situation occurred--that is, gravicurvature without concurrent auxin redistribution--were observed. Collectively, these results indicate the following sequence of key events in shoot

gravitropism: gravity perception ⟶ auxin redistribution ⟶ asymmetric acid efflux ⟶ asymmetric growth.

During 1983, there has been increasing interest in the possibility that calcium may play a role in the gravitropic response of plant organs. Three kinds of observations tend to support this view: (1) asymmetric Ca^{++} movement has been detected in gravistimulated roots and shoots, (2) application of a Ca^{++} gradient can initiate curvatures even in the absence of gravity stimulation, and (3) Ca^{++} chelators such as EGTA can stimulate straight growth and, when applied asymmetrically, initiate gravitropismlike curvatures. In the latter part of 1983, studies focusing on the mechanism by which EGTA stimulates shoot growth were begun. As this chelator is being used extensively in tropism-related studies, it was considered important to know if indeed its growth-promoting activity derived from removing wall-bound Ca^{++} or by some unrelated phenomenon.

Using Avena coleoptile sections, it was confirmed that EGTA does indeed stimulate growth provided the sections are abraided to remove the cuticle. However, during the course of these studies it was also observed that at neutral pHs EGTA liberates protons (from the nitrogens) as it binds Ca^{++}. As protons are well known to stimulate growth, it seemed possible previous workers were simply observing acid-promoted growth in response to EGTA application--a phenomenon which would have no direct implications regarding Ca^{++} and the physical properties of plant cell walls. This possibility was tested using a model system employing frozen-thawed coleoptile sections and an external force to simulate turgor. Data indicate removal of Ca^{++} from cell walls by EGTA does not in itself stimulate cell extension. Rather, it can be shown that EGTA-induced growth is indeed simply due to wall acidification and subsequent acid growth. In light of this finding, extreme caution is needed in evaluating the effect of EGTA on root and shoot gravitropism as the observed responses may be indirect and thus misleading.

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THE CELLULAR BASES OF GRAVITY- AND LIGHT-INDUCED GRAVITROPISM

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Light greatly accelerates the positive gravitropic response of certain roots and alters the gravitropic sensitivity of coleoptiles and stems. Thus, it is generally believed that some cellular response initiated by light is the same as, or affects, one of the gravity-induced cellular responses necessary for gravitropism. The objective of this research is to elucidate the specific fundamental processes that are altered by gravity and light in the induction of gravitropic growth in plants.

Previous results have suggested that at least one of these fundamental processes is the movement of calcium ions in the stimulated cells and organs. Calcium ions have repeatedly been shown to play an important role in the regulation of plant growth and development, and both gravity and light induce calcium fluxes in responding tissues. During 1983, research was focused on several major questions pertinent to the proposed role of calcium in gravity- and light-induced gravitropism: (1) What is the subcellular distribution of the calcium-activated regulatory protein calmodulin in plant cells? (2) Can calcium chelators alter the gravitropic responsiveness of plants in a specific fashion? (3) Does one observe a rapid redistribution of calcium in roots, as is found in shoots, early during the induction of gravitropic growth?

At least some calcium-mediated responses in plants are controlled by the calcium-binding regulatory protein, calmodulin. Earlier published reports of ours had indicated a possible role for calmodulin in mediating light and gravitropic responses in oat coleoptiles. To help further evaluate how calmodulin could affect plant growth, calmodulin was isolated and characterized from oats and its content was estimated, both in intact tissue and in isolated subcellular fractions, by radioimmunoassay. The results indicated that calmodulin is present in large quantities in plant cells and that it is specifically associated with mitochondria, etioplasts, and nuclei. In an analysis of a specially prepared extract of soluble wall proteins, calmodulin was found to be the major component of these proteins, too. If further research conclusively demonstrates that calmodulin is indeed a wall protein, this would provide a plausible regulator target for the calcium which moves asymmetrically into walls early during gravitropism.

The calcium chelator EGTA may be used as a probe to test whether calcium movements are important for gravitropism, since it could

both chelate wall calcium and possibly inhibit the activation of calcium-dependent plasma membrane pumps. The research group of M. Evans, funded by NASA, found that application of EGTA to the tips of corn roots caused a loss of gravitropic sensitivity, which could be restored by replacing the chelator with calcium chloride. The effect of EGTA on the gravitropism of etiolated oat coleoptiles was investigated. It was found that a treatment period as brief as 2 hr. in 1 mM EGTA completely blocked gravitropism in 50-60% of the treated coleoptiles without inhibiting growth. Only about 10% of the plants perfused in water failed to exhibit gravitropism. Subsequent perfusion of EGTA-treated plants with calcium completely restored gravitropism; postperfusion with water did not. Controls showed that the effects of EGTA could not be attributed to its weak capacity to buffer the pH of the treatment solution. The fact that calcium reversed the inhibitory effects of EGTA on gravitropism indicated that the inhibition was probably due to a reduction in the availability of free calcium required for one or more of the transduction steps of gravitropism.

As of 1983, it is not known whether a gravitropic stimulus induces a redistribution of calcium in roots as it apparently does in coleoptiles and shoots. The work of Evans on the inhibition of root gravitropism by calcium chelators, mentioned above, and his finding that calcium transport through root tips is altered early during root gravitropism suggested that calcium movements might be important for root gravitropism. This led to an investigation of whether a gravitropic stimulus of corn roots would result in a redistribution of calcium in this organ and, if so, how soon and in what cells would this redistribution occur. Antimonate staining procedures were used to determine the patterns of localization of calcium in nonstimulated and gravistimulated corn roots. It was found that in the region of the developing bend there was a change in the staining pattern from that principally localized within cells of the stele to asymmetric staining within the vacuoles in the cortical cells along the upper surface of the root. There was very little staining in the walls. This staining pattern is quite different from that seen in gravistimulated coleoptiles, but it does represent an asymmetric redistribution of calcium induced by a gravitropic stimulus, and it does occur rapidly enough (within 10 min.) to play a potential role in the regulation of root gravitropism which becomes visible only after about 20 min. This question needs to be investigated further.

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GRAVITROPISM IN LEAFY DICOT STEMS

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Objectives

(1) To understand mechanisms of gravity perception.
(2) To consider the possible role of plant growth substances in transduction of the perceived stimulus to actual bending.
(3) To study the mechanics of stem bending: kinetics of the process, the forces involved, the mechanical properties of cell walls, etc.

Salisbury spent most of 1983 on sabbatical in Austria and Israel. This report encompasses his research plus that of two students in Utah.

The Mechanics of Gravitropic Stem Bending. Cocklebur (Xanthium strumarium) stems were subjected to four treatments: (1) horizontal restrained stems (stem placed between two stiff wires and wrapped with thread), (2) horizontal free bending, (3) vertical controls, and (4) stems restrained for 48 hr. or more and then released by cutting the threads. Stems bent immediately, typically to about 135°. Both tops and bottoms of horizontal stems were photographed with two cameras (stereo photogrammetry). Length measurements were taken between dots of india ink on stems by projecting the negatives onto a digitizer interfaced with a microcomputer.

Growth nearly stops on the top of horizontal free-bending stems and is accelerated on the bottom compared with controls. In the bending regions on the top, there is often a significant shrinkage. Upon release of a horizontal restrained stem, the top shrinks considerably, and the bottom elongates, emphasizing the cessation of growth in upper tissues when stems are turned to the horizontal. (Wesley Mueller, completed Ph.D.)

When a vertical stem is split from the tip toward the base, the two halves bend away from each other, indicating that internal stem tissues are under pressure compared with surface tissues. This was studied in castor bean (Ricinus communis) by making horizontal cuts at 2.5-mm. intervals halfway around one side of the stem. Depth of the cuts was controlled by the extent to which a razor blade protruded from between two pieces of a holder and was measured on free-hand sections after the degree of bending (always away from the side of the cuts) had been measured. Plotting stem bending as a function of depth of the cuts showed that maximum bending was achieved when the cuts were about 0.6 mm. deep, extending through epidermis, collenchyma, cortex, and at least into the phloem of the vascular bundles.

Thus, pressure seems to be localized in the pith (even in these hollow stems), and tensions apparently exist in all the remaining tissues.

A device was built to cut longitudinal sections of measured thickness from the center of plant stems, giving increased bending of the two halves (usually equal for a vertical stem). When horizontal stems are bending upward, the upper half of the cut central section bends about 20° more than the lower half, emphasizing the importance of the gradient in tissue tension/pressure in the upper half of free-bending stems. Split central sections were placed in sorbitol solutions of varying concentrations. Sections placed in solutions hypotonic to the cell sap continued to bend as pith cells took up water, while hypertonic solutions caused an inward bending (a straightening) of the two stem halves. Response in solutions close to isotonic suggest that water potential in the bottom pith is less negative than in the top. (Salisbury)

Effects of Unilateral Applications of Ethephon. Solutions of ethephon were painted on one side of stems and hypocotyls of young tomato (Lycopersicon esculentum), cocklebur, castor bean, and sunflower (Helianthus annuus) plants. Vertical stems and hypocotyls (especially tomato) bent away from the side of application within 2-3 hr. When ethephon was neutralized to pH 6, vertical stems did not bend away from the side of application, but leaves developed epinasty, a typical ethylene response. When buffered acid or even unbuffered HCl was applied to one side of vertical stems, bending occurred away from the side of application, epinasty did not develop, but stems were damaged in a manner similar to that produced by unbuffered ethephon.

When ethephon (pH 6) was applied to the bottom of stems just turned to the horizontal, gravitropic bending was delayed. Acid solutions applied to the bottom of horizontal stems greatly delayed bending--an unexpected result in light of the experiments just described. Ethephon (pH 6) applied to the upper surface of horizontal stems caused a small delay in gravitropic bending, but acid solutions applied to the top of horizontal stems caused downward bending. (Rosemary White)

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ANIMAL PROJECTS

THE EFFECT OF SKELETAL UNLOADING ON BONE FORMATION

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Available data suggest that bone mass is a result of the balance of bone formation and bone resorption. Skeletal unloading as occurs in spaceflight, bedrest, or immobilization affects this balance and leads to osteoporosis, negative calcium balance and osteopenia. A role for both hormones and local factors in maintaining this balance seems likely, but it is not clear to what extent skeletal unloading alters the balance through effects on hormones vs. local factors. A model in which growing rats are suspended so that their hind limbs are unweighted but their forelimbs are normally weighted is being used to test the role of hormones in mediating the local disturbances in bone formation caused by selective skeletal unloading.

Growing rats (160-200 g) have been suspended by their tails for at least 4 wk. without altering their growth patterns. No difference in weight gain between such animals and their pair-fed controls was observed. The suspended rats in comparison to the pair-fed controls showed a decrease in fat free weight, ash weight, and calcium content of the unloaded bones (tibia, lumbar vertebra) but not of the normally loaded bones (humerus and mandible). Most of the difference in bone mass between suspended and pair-fed controls occurred progressively between days 2-10, after which no further difference between suspended and pair-fed controls was observed. The effect of suspension on two biochemical measures of bone formation--^{45}calcium uptake presumably into mineral and ^{3}H-proline uptake presumably into matrix--has been examined. The proximal portion of the long bones, which contains a higher percentage of endosteal bone than the shaft (which is primarily cortical bone and has a higher percentage of calcium/mg fat free weight) had a 3-fold greater amount of ^{45}Ca and ^{3}H proline incorporation. With suspension, both portions of the tibia showed a decrement in calcium content, calcium uptake, and proline incorporation by 5-7 days when compared with the tibia from pair-fed controls. After 10 days, no further decrement in calcium content was seen and ^{45}calcium and ^{3}H-proline uptake rebounded to control or above control levels. The bone formation rate was determined at the tibia-fibula junction (cortical bone) in suspended rats and pair-fed controls using three tetracycline labels administered on days 3, 9, and 14 to mark two periods of bone formation: period 1 = days 3-9, period 2 = days 9-14. The results indicate that suspension unloading leads to a 50% reduction in bone formation

in the tibia during period 1, but with continued suspension these measurements of growth return to normal.

The initial approach to determining whether the calciotropic hormones were involved in signaling the unloaded bone to stop growing was to measure calcium, phosphorus, PTH, and $1,25(OH)_2D$ in the serum of suspended and pair-fed control rats. The results indicate no differences in the serum levels of any of these ions or hormones at 15 days. However, small increases in serum calcium were observed at days 5-7 of suspension which returned to pair-fed control values by day 15. The rise in serum calcium was associated with a fall in serum $1,25(OH)_2D$ values. Furthermore, a small reduction in intestinal calcium transport (a process regulated by $1,25(OH)_2D$) occurred between 5-10 days of suspension which returned to control levels by day 15. These observations suggest that cyclic changes in the serum levels of the calciotropic hormones during skeletal unloading may provide at least part of the mechanism by which bone formation is inhibited by day 5 and is restored to normal by day 10.

These data are also consistent with the possibility that skeletal unloading alters the response of bone cells to calciotropic hormones. Such an alteration in bone cell response would explain why bone cell formation ceases in the tibia but not in the humerus of the suspended rat. An attempt is currently being made to determine whether the receptors for $1,25(OH)_2D$ and PTH in bone are altered by skeletal unloading. An assay to measure $1,25_2D$ receptors in the cytosol extract of pulverized bone from which marrow has been carefully removed has been developed. The $1,25(OH)_2D$ receptor from bone is similar to the $1,25(OH)_2D$ receptor from other tissues in that it is 3.7S in size and specific for $1,25(OH)_2D$.

Finally, isolation of bone cells from the long bones of the growing rat has just begun. Initial experiments indicate that approximately 10^6 cells can be isolated per bone (tibia, femur) which in culture develop into typical appearing bone cells. In future experiments, such preparations will be tested for their biologic function and response to PTH and $1,25(OH)_2D$ in an effort to evaluate whether cells obtained from a bone in which formation has been inhibited by tail suspension differ from cells obtained from a bone in which bone formation is unimpaired.

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WEIGHTLESSNESS SIMULATION: PHYSIOLOGICAL CHANGES IN FAST AND SLOW MUSCLE

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Weightlessness during spaceflight produces numerous physiological changes in virtually all organ systems. Particularly affected is the musculo-skeletal system. Changes in bone turnover and muscle atrophy have been well described. So far, however, there have been no systematic reports available based on biochemical, physiological, and pharmacological approaches to studying the cholinergic system of nerve and muscle during hypokinetic conditions. The key enzymes, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), involved in the synthesis and hydrolysis of neurotransmitter, acetylcholine (ACh), are reported to be synthesized in the cell bodies of cholinergic motor neurons which innervate skeletal muscles. Following synthesis of both these enzymes, they are transported by axoplasmic flow to nerve terminals. ChAT is thought to remain highly localized in nerve terminals with low presence in the muscles proper, whereas, AChE is present in the axon in equally high or higher concentration than in the muscle. Its presence in muscle depends to a large extent on innervation.

The transmission of impulses from nerve to muscle is caused by the release of ACh from nerve terminals which interacts with the nicotinic ACh receptor in the muscle fiber. Only the endplate membrane directly beneath the nerve terminal is highly sensitive to ACh and also has the highest density of ACh receptors. Denervation causes the entire muscle fiber to become sensitive, while reinnervation restricts this ACh sensitivity once again to the endplate membrane.

The overall aims of this proposal are directed to study alterations in muscle biochemical, morphological, and physiological characteristics as they are produced by simulated weightlessness and the time course of recovery after release from weightlessness.

During 1983, the research has focused on three major questions. (1) What is the effect of disuse on AChE activity in muscle and peripheral nerve and how does it affect the regulation of the molecular forms, 4S, 10S, 12S, and 16S, in slow and fast muscle. (2) How is the enzyme that regulates acetylcholine (ACh) synthesis, i.e., ChAT, affected in nerve and muscle. (3) What are the effects of prolonged disuse on the ACh receptor in slow and fast muscle. (4) How does prolonged disuse affect morphological as well as biochemical characteristics in slow and fast muscle?

To produce experimental weightlessness, rats were suspended by their tails in such a way that their hind legs were completely unloaded, while the animals were able to move freely on their forelegs throughout the cage. Animals were suspended from 1 to 3 wk. Activities of ChAT and AchE were measured with highly sensitive radiochemical assays, and the molecular forms were separated by velocity sedimentation and quantitated with the radiometric assay. To study the quantitative changes in ACh receptors ^{3}H-ACh was used as the natural ligand for nicotinic receptor sites.

With disuse induced by hind limb suspension, the predominantly slow soleus (SOL) lost 60% of wet weight while the fast extensor digitorum longus (EDL) lost 29% compared with their respective control muscles. The activity of ChAT was markedly elevated in sciatic nerve (38%), soleus, and EDL (79% and 78%, respectively) compared with controls. The increase in ChAT activity was time-dependent in both muscles as the maximal change was noticed at the end of 3 wk. In soleus, AChE activity increased by 250% involving all molecular forms such as 4S, 10S, 12S, and 16S forms, while there was only a minimal change in the EDL. Total protein concentrations in nerve and muscle were not significantly different in control and hind limb suspended animals. By using ^{3}H-ACh as the ligand for the nicotinic receptor, there was evidence of increased nicotinic AChR binding in both SOL and EDL muscles. Fiber typing by actomyosin ATPase reaction showed a shift in fiber type profile in the soleus towards a larger proportion of fast (Type II) fibers.

It is evident from the present findings that some properties of skeletal muscles are strongly dependent on pattern and level of load-bearing and motor unit activity. The antigravity muscle such as the SOL appears to be more affected by hind limb suspension than the fast EDL. Whether all the observed changes are due to fiber type changes, as observed in SOL, remains to be seen.

Future studies will investigate the effects of weightlessness on axoplasmic transport, the electrophysiology of neuromuscular transmission, and the reversibility of the observed changes during recovery from hind limb suspension.

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ALTERATIONS IN CONNECTIVE TISSUE CELLS DUE TO REDUCED WEIGHT BEARING

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The research objectives are: (1) to determine and describe effects that occur to connective tissue cells as a result of reduced gravitational or mechanical forces, and (2) to increase the understanding of normal cell function by perturbing normal gravitational or mechanical forces and searching for mechanisms that will describe the cellular behavior.

The cells being studied are derived from skeletal, cartilage, and dense connective tissues. Tissues have been collected from rats subjected to spaceflight conditions (e.g., Cosmos 1129) and from animals placed under non-weight-bearing conditions in a 1-G environment (the Morey-Holton suspended animal model). The methods of cell study include light, fluorescence, and electron microscopy, autoradiography, histochemistry, and some biochemical measurements.

Results that have been obtained relating to Objective 1 indicate: (1) An extracellular cell membrane-associated alkaline phosphatase enzyme has shown reduced activity as a result of reduced mechanical stress. (2) Golgi-associated enzymes have reduced activity in those cells actively synthesizing-collagen. (3) Lysosomal enzyme activity is increased in collagen-synthesizing cells, evidently in response to less need for matrix collagen. (4) Cell-cell communication maintained through special junctional complexes between cells appear to be reduced in size and number as a result of reduced weight bearing or hypogravity conditions.

These studies are continuing in order to determine whether these results are possibly due to some systemic factor (e.g., a change in circulating hormones, altered blood flow, production of inhibitory substances, or other unknown blood-borne factors), or whether the cellular changes are a direct response to altered mechanical forces.

During 1983 work began on Objective 2. It has been found that a calcium-stimulated ATPase can be localized in bone cells, which appears by light microscopy to be within the cell cytoplasm and may be located at the site of junctional complexes. This would be exactly opposite to the localization of membrane-associated alkaline phosphatase. In fact, electron microscopy shows that alkaline phosphatase is on the extracellular surface of the cell

membrane and does not exist at the site of junctional complexes. It was also noted that calcium ion at 2-5 mM produces the greatest ATPase activity whereas 10-mM calcium inhibits this enzyme. The interrelationship between ATPase and alkaline phosphatase is not yet known. However, there is now the exciting possibility that an ATPase is regulated by calcium ion and a phosphatase is controlled by phosphate ions. Thus, mechanisms exist for controlling cellular activity through changes in environmental ion products.

In addition to stimulation of ATPase, calcium ion also influences the cytoskeleton of connective tissue cells. This cytoskeleton functions importantly not only in mechanisms of cellular secretion but also in the regulation of cell motility. Therefore, by fluorescence localization of fibrous actin, it has been determined that actin content of bone cells has been reduced as a result of skeletal non-weight-bearing. The next step is determining whether the actin content and cytoskeletal or microfilament contractility is controlled by calcium ion levels or by direct transduction of external mechanical force across cell membranes into the cytoskeletal network. These studies will provide important insight into how environmental factors may exert control over normal cell functions.

GROWTH AND DIFFERENTIATION OF MAMMALIAN EMBRYONIC TISSUES EXPOSED TO HYPERGRAVITY IN VIVO AND IN VITRO

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The specific aims of this research are (1) to determine the effects of centrifugation on the cytology and biochemistry of differentiating systems; (2) to ascertain whether a quantitative relationship exists between gravitational force and development; (3) to identify gestational ages and basic cellular processes susceptible to gravitational forces; (4) to determine the duration of exposure needed to effect a change; and, (5) to investigate the effectiveness of the clinostat as a zero G simulator in a mammalian system.

The in vitro studies have shown that excess gravity (2.6 G) is able to suppress morphogenesis in embryonic mouse limbs developing in vitro by promoting early differentiation. These earlier studies were carried out with BGJ_b medium containing 25% fetal calf serum and have been repeated with serum-free medium (BGJ_b plus 25% salt solution) to insure that the changes seen in centrifuged limbs are not due to sedimentation of necessary serum components. Morphogenetic scoring of the various elements in fixed whole mounts of control limbs showed that the serum-free medium supported limb development just as well as did medium with serum. Also, centrifuged limbs attained scores similar to those of centrifuged limbs in the previous study, showing that the possible unavailability of serum components involved in growth regulation was not a factor in the lower morphogenetic scores seen in centrifuged limbs. Other observations seen in the previous study were repeated: (1) there is a proximo-distal gradient of sensitivity to gravity's teratological effects with distal elements (i.e., less differentiated ones) being more susceptible and proximal ones less so; (2) only certain stages in limb development exhibit geosensitivity and these stages are the same as for other (i.e., chemical) teratogens; (3) centrifugation may cause limb elements present at explantation to resorb; and (4) finally, the lack of morphogenesis seen in centrifuged limbs is due to early differentiation of cells.

In addition to the cytologic and morphometric studies, histochemical analyses of excess gravity on chondrogenesis have been carried out on micromass cultures of embryonic mouse limb cells using type-specific anticollagen antibodies and various immunological techniques (HRP, fluorescence).

The major biochemical transitions that occur during cartilage

differentiation are the initiation of synthesis and disposition of macromolecules of the cartilage-specific extracellular matrix. Therefore, the studies also propose to characterize transitions in phenotypic expression of differentiating limb buds in chondrogenic stages using biochemical markers such as chondroitin sulfate and collagen type.

For biochemical studies, the limb buds are rinsed, blotted, lyophilized, and then weighed to obtain dry weights. Lyopholized samples are dissolved in 0.1N NaOH prior to determination of collagen, glycosaminoglycan, and noncollagen protein accumulation. The initial results of 12-day limb bud cultures indicate that no significant difference in content of collagen is exhibited by exposed vs. nonexposed limb buds, but a 23% decrease in glycosaminoglycan concentration is seen in limbs developing under excess gravity.

Recently, the effects of exposure to excess gravity on fusion of the embryonic mouse secondary palate have been studied. During fusion, the palatal shelves first adhere by means of glycoproteins appearing along the medial epithelial edge. The contacting epithelia then reorganize and undergo programmed cell death, allowing the underlying mesenchymes to come in contact. The process of cell death occurs *in vitro* at about the same time that it occurs *in vivo*. When embryonic palates were exposed to excess G *in vitro* for 24 hr., and then examined by light and electron microscopy, palatal cell death and fusion were accelerated under 2.6 G.

With the recent completion of a small animal centrifuge examination of the effect of hypergravity on *in utero* mammalian development is now possible. The centrifuge is able to house up to 80 mice, 20 at each of four different gravities. The animals are provided with chow *ad libitum* and water is provided through a nuzzle valve system. At present, 60 young mice are undergoing adaptation prior to mating, having been placed on the centrifuge at 4 wk. of age. Control animals are housed in the centrifuge room.

Besides the *in vivo* and *in vitro* centrifuges for excess gravity studies, a clinostat is being developed for zero G studies.

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EFFECTS OF GRAVITO-INERTIAL FIELDS ON THE PHYSIOLOGY OF THE ORB-WEAVING SPIDER

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This project is directed toward identifying the gravity receptor of the spider and evaluating its role in the maintenance of equilibrium. Prior to this NASA-supported work, gravity and the spider had not been studied. This research also evaluates the operating characteristics of the orb-weaving spider: the absolute threshold to hyper-Gz and the dynamic range of increments to gravity. Improvements in experimental methodology and data analysis during 1983 have permitted the investigation of issues in gravitational physiology of the spider heretofore not possible.

The initial work on hypergravity produced by centrifugation indicated a system having a dynamic range of sensitivity of at least four orders of magnitude in which the absolute threshold to hyper-Gz was less than 1.001. In the first experiments it was necessary to measure Gz effects postrotation. During 1983 an "on-board" system, employing a laser diode, permitted the cardiac reflex to be taken during centrifugation. Replication of the postrotation study confirms the logarithmic relationship between the response of the spider heart and Gz intensity, as well as the low absolute threshold to increases in gravity.

During the year the centrifuge drive system was modified so that it is now under computer control. Thus, small or large decelerations or accelerations, with exposure time held constant or systematically varied, can be programmed. An issue that can now be attacked directly concerns the differential sensitivity of the spider to changes in Gz (delta Gz). The procedure can be viewed as producing a Corioluslike stimulus, with the animal remaining motionless but the gravito-inertial field changing in magnitude over highly controlled time periods. Its good sensitivity to constant radial acceleration suggests that the spider response to delta Gz might also be a small value. Most probably the Weber Ratio (delta Gz/Gz) is a relatively constant value for low intensity gravito-inertial fields. This aspect of the research is just beginning.

The lyriform organ on the spider legs has been identified as a source of vibratory and proprioceptive information. It apparently has a dual role in sensory transduction, providing the animal with relevant cues as to the magnitude and direction of the Gz stimulus. Removal of the patellar lyriform organs results in a decrease of sensitivity to Gz and a statistically

significant increase in the rate of the cardiac response. Small changes in Gz and/or in tilt are also accompanied by a reduction in the rate and/or amplitude of the beat over a period of time. The recovery time from this bradycardia is not yet known. Thus the lyriforms are involved with Gz detection and there is an indication that they may provide an inhibitory input to the neural cardiac pacemaker. During 1983, a brief communication from another laboratory reported that the spider heart was innervated by neurons traversing the abdominal nerve possibly arriving from the suboesophageal ganglion (SOG). Within the SOG are the centers that integrate the sensory input from the lyriform organs. These may be the morphological bases for observations that sensory stimuli can produce a bradycardia of the spider heart. This is important because of postulations that the neurogenic heart is an active element in the spider equilibrium system. That is, blood serum pumped into the cephalothorax can produce leg extension and the spider is supported under changed gravity conditions.

A test of the hypothesis that the lyriform organs exert inhibitory control on the neurogenic heart can be made by substituting a vibratory stimulus for the Gz stimulus. If, as is supposed, the lyriform encodes both vibration and gravity, then a small displacement of the legs should inhibit the cardiac response. In the latter part of 1983 the spider heartbeat was recorded by laser-plethysmograph with animals placed on a vibration table. A variety of stimulus frequencies (from DC to about 150 Hz) delivered at suprathreshold amplitudes (circa less than 1 micrometer) produced bradycardias. This is evidence that, in the spider, gravity and vibration have a similar physiological effect. It also implies that the spider contains a frequency analysis mechanism for discriminating the stimulus input.

The relationship between gravity/vibratory stimuli and the beat of the neurogenic heart of the spider seems clear. If the mechanical input serves to inhibit cardiac activity then in the microgravity of orbital flight the heart should beat at an inordinately high rate, but sensory control should be reinstated by vibration of the legs. An application to test this hypothesis during a "shuttle flight" has been submitted to the NASA Space Biology Program.

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HOMEOSTASIS IN PRIMATES IN HYPERACCELERATION FIELDS

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The homeostatic capabilities of animals are sensitive to changes in the ambient acceleration environment. Such changes in centrifuged rats and dogs include depressed body temperatures, alterations in circadian timekeeping, and changes in body composition. To date, however, little work has been done examining these changes in man or any other primate. During 1983, various homeostatic responses of a nonhuman primate, the squirrel monkey (Saimiri sciureus), to acute changes in the acceleration environment were examined. When these animals were exposed to a hyperdynamic field, the body temperature was consistently depressed and the animals showed behavioral indications of increased drowsiness. Further, time of day played a significant role in influencing these responses.

Initially, loosely restrained squirrel monkeys were exposed to hyperdynamic fields. The centrifugation consisted of a 60-min. step change in the acceleration environment from 1 G to 2 G in the z axis (head-to-toe). All animals demonstrated significant depressions of body temperature while in the hyperdynamic field. After the centrifuge was started, there was generally a small increase in body temperature. Within 5-10 min., however, a continuous decline in temperature became apparent. The average decrease in body temperature was 1.4° C and the body temperature depression was maintained for the duration of the step change. Approximately 5 min. after the animal was returned to 1 G the body temperature began to rise.

Next, the primate sleep responses to 70-min. exposures to the 2 Gz hyperdynamic fields were examined. Initial observations on centrifuged animals, when observed visually, suggested a sleeping behavior when in the hyperdynamic field. This phenomenon was examined further with electrophysiological recording of the EEG in a group of chronically implanted primates. During the precentrifugation phase, the animals showed various amounts of "napping" behavior in which slow wave sleep occurred on a periodic basis. (Rapid eye movement sleep was never observed during this daytime study.) Upon centrifugation, however, sleep was inhibited for several minutes after which the slow wave sleep pattern began to recur. The amount of sleep never reached the baseline level of the precentrifugation phase. Postcentrifugation, the amount of sleep increased significantly and was maintained above baseline levels for the entire 70-min. postcentrifugation phase. This response was proportional to field intensity in the animals studied at both 2 and 3 G_z. At 3

Gz, the animals showed an increased response in that there was a greater rebound in slow wave sleep during the postcentrifugation phase. It is interesting to note that slow wave sleep has always been inversely correlated with body temperature. That is, lower body temperatures are usually associated with increased sleep. In this experiment, the correlation did not hold. During centrifugation, the amount of sleep observed during the fall in body temperature was less than that seen in the control phase where body temperature was higher.

Finally, the thermoregulatory responses of squirrel monkeys to 70-min. 2-Gz exposures during the day and night were compared. Again, the response of the animal during the day was, on average, a 1.5^{o} C fall in colonic temperature, mediated at least in part by an increase in vasomotor heat loss. This response was highly reproducible and seen in all animals. In contrast, at night the temperature response was completely eliminated. Individual animals showed small deviations in temperature either in an upward or downward direction, but on the average there was no change in colonic temperature. Similarly, the skin temperature did not show any major deviations in vasomotor heat loss. These results demonstrate that although the centrifuge-induced changes in body temperature are very prominent and reproducible during the day, these animals can regulate their body temperature independently of the gravitational field during the night.

This day-night difference in body temperature response was also investigated in a series of rats. Historically, all rats exposed to hypergravitaitonal fields have been examined during the day, which is the rest phase of these nocturnal animals. An attempt was made to determine whether the light vs. the dark had a significant influence on the response or whether the magnitude of the response was a function of the activity level of the animal. The previously reported 2^{o} C response was observed when centrifuging the animals during the day. However, upon exposure to 2 Gz at night, which is the animal's active phase, the body temperature fall was even larger. This indicates that although the monkeys were able to regulate body temperature more precisely than the rats, both species showed an increased ability to maintain body temperature during their rest phase when their circadian body temperatures were at a minimum.

Current efforts are proceeding to examine the influence of chronic acceleration on these and other homeostatic systems, with special interest in examining the new steady-state levels of these homeostatic variables and their 24-hr. rhythmicity. Finally, protocols are being developed to further describe the mechanisms by which these responses are produced.

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NEURAL MECHANISMS BY WHICH GRAVITATIONAL STIMULI AND STRESS AFFECT THE SECRETION OF RENIN AND OTHER HORMONES

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This project was activated August 1, 1983. The goal of the research is delineation of the neural pathways and transmitters that mediate the changes in the secretion of renin and the antidiuretic hormone produced by gravitational and other stimuli. Evidence indicates that stimulation of serotonergic neurons in the dorsal raphe nucleus of the midbrain increases renin secretion, and that these neurons project to the mediobasal hypothalamus (Karteszi, et al., Neuroendocrinology 34: 323-326, 1982). The initial goal of the present project has been to determine how the message gets from the hypothalamus to the renin-secreting cells in the kidneys.

A pharmacological study was undertaken to determine if the pathway from the hypothalamus to the kidney was sympathetic. Catecholamines secreted from the renal nerves and the adrenal medulla are known to mediate sympathetic stimulation of renin secretion via beta-adrenergic receptors located on the renin-secreting cells. The serotonin-releasing drug p-chloroamphetamine (PCA) was used to initiate serotonergic discharge in rats and the sympathetic effector pathway was blocked by administration of the beta-adrenergic blocking drug L-propranolol. The D isomer of propranolol, which has all the properties of the L-propranolol except that it has only weak beta-adrenergic blocking activity, was used as a control. L-propranolol but not D-propranolol prevented the increase in plasma renin activity produced by PCA. Because propranolol penetrates the brain, and it could be argued that it was exerting its effects on the brain rather than the kidney, the experiment was repeated with sotalol, a beta-adrenergic blocking drug which does not cross the blood-brain barrier. Sotalol also prevented the renin response to PCA.

In other experiments, the effects of PCA were tested after transmission in sympathetic ganglia was blocked by administration of the ganglionic-blocking drug, chlorisondamine. Chlorisondamine increases plasma renin activity by itself because it interrupts the tonic sympathetic discharge to the blood vessels that maintain blood pressure. The resultant decrease in blood pressure acts directly on the kidneys to increase renin secretion. However, PCA plus chlorisondamine produced no greater increase in plasma renin activity than chlorisondamine alone. Failure of plasma renin activity to increase was not due to renin secretion being maximal after chlorisondamine because a further

increase in renin secretion could be produced by injecting isoproterenol, a drug that bypasses the block to act directly on the beta-adrenergic receptors on the renin-secreting cells in the kidneys. Thus, both the beta-adrenergic blocking experiment and the ganglionic-blocking experiment indicate that the renin-stimulating effect of serotonergic discharge is mediated via the sympathetic nervous system. A paper reporting these results is in press.

Detailed investigation of the parts of the hypothalamus and brainstem involved in regulating the renin response to PCA and other stimuli have now begun. Discrete lesions are being produced in various parts of the hypothalamus and the brainstem and the response to PCA is being tested. These experiments will subsequently be expanded to determine the effects of these lesions on the renin response to tilting and psychological stimuli.

Some preliminary experiments, have also been conducted to see under what conditions head-up tilting, one of the gravitational stimuli to be used in experiments, increases plasma renin activity. Preliminary experiments aimed at discovering a reliable and reproducible psychological stress that increases renin secretion in rats are also underway.

CHANGES IN BONE STRUCTURE AND METABOLISM DURING SIMULATED WEIGHTLESSNESS: ENDOCRINE AND DIETARY FACTORS

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The objective of this research program is to determine the role of specific endocrine factors in the bone changes associated with simulated weightlessness and at the same time investigate the dynamics of bone metabolism. A rat model, in which the animal is suspended by its tail, is used to simulate weightlessness. Specific objectives were to: (1) determine the relationships between the circulating concentrations of calcium (Ca), inorganic phosphorus (P_i), parathyroid hormone (PTH), 1,25-dihydroxyvitamin D (1,25 $(OH)_2D$), and the bone changes induced by simulated weightlessness; and, (2)determine the temporal changes in the serum concentration of 1,25$(OH)_2D$ following suspension.

The first objective was accomplished by manipulation of dietary Ca and P. Four diets were used ranging from low Ca (0.1%), normal P (0.3%) to high Ca (2.4%), high P (1.2%). The animals were studied after 2 wk. of suspension (simulated weightlessness). The results of these experiments indicate that the bone mineral loss which occurs during simulated weightlessness on a diet with normal levels of Ca and P can be reduced by increasing the dietary intake of these elements. On a normal diet, tibia from animals undergoing simulated weightlessness showed a highly significant ($P<.005$) 13% decrease in total weight when compared with tibia from control animals. On a high Ca/P diet no significant differences in bone weight were observed.

The serum concentrations of Ca, P_i, PTH, and 1,25 $(OH)_2D$ were virtually identical in suspended and pair-fed control rats on all diets except the high Ca/P diet. In this case, serum P_i was elevated in suspended rats (6.0 $\pm$ 1.5 mg/dl vs. control of 4.8 $\pm$ 0.6 mg/dl) and serum 1,25 $(OH)_2D$ was depressed (66 $\pm$ 18 pg/ml vs. control of 112 $\pm$ 19 pg/ml). The similarities (except in the case of the high Ca/P diet) in the mineral and hormonal composition of serum from suspended and control animals after 2 wk. of suspension seem to suggest that changes in the circulating concentrations of PTH, and 1,25 $(OH)_2D$ are not responsible for the differences in bone mineral content observed at this time. However, later studies, which examined bone formation rates (uptake of ^{45}Ca into the bone) as a function of time after suspension, indicated that bone formation falls dramatically during the first week of suspension and then returns to approximately normal during the second week. At 14 days of suspension, even though there is less total bone mass, formation

is nearly normal. This is consistent with the fact that serum concentrations of Ca, P_i, PTH, and 1,25$(OH)_2D$ (with the exception of the high Ca/P diet) are also normal.

To examine the relationship between bone formation and the serum concentrations of Ca, P_i, PTH, and 1,25 $(OH)_2D$ (the second objective) during the early phases of suspension, animals were sacrificed at 2, 5, 7, 10, 12, and 15 days after being suspended. Although not all data are available (PTH has not yet been measured), the results indicate that suspension causes a transient hypercalcemia and a transient fall in the circulating concentration of 1,25 $(OH)_2D$ from 130 $\pm$ 13 pg/ml to 50 $\pm$ 12 pg/ml. This occurs between days 2-7 of suspension and is followed by normalization of both variables. At the same time that animals are hypercalcemic and have low serum concentrations of 1,25 $(OH)_2D$, bone formation is inhibited.

These findings can be interpreted in at least two ways. On the one hand, suspension may cause a transient inhibition of bone formation (primary defect). The reduction in Ca demand by the bone may in turn cause a transient hypercalcemia which could lead to a transient reduction in circulating 1,25 $(OH)_2D$ concentration. On the other hand, the primary defect may be a reduction in renal 1 alpha-hydroxylase activity, the enzyme primarily responsible for maintaining the serum concentration of 1,25 $(OH)_2D$. A reduction in serum 1,25 $(OH)_2D$ may reduce bone formation which in turn could cause a transient hypercalcemia. Further experiments are presently underway to examine both of these hypotheses.

THE EFFECTS OF HYPERGRAVIC FIELDS ON NEURAL PROCESSING OF SENSORY THERMOREGULATORY AND VESTIBULAR SYSTEMS IN THE RAT

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A major objective during 1983 was the continuation of studies on temperature regulation in mammals exposed to hypergravic fields. Experiments involved measurement of the rate of oxygen consumption and core and tail temperatures in cold-exposed rats in hypergravic fields from 1.5 to 6 G. One series of studies was completed, and additional experimental studies are partially completed. Data obtained during 1983 from the ongoing studies include the following:

First, heat conservation and production were studied in rats at 3 G and at 1 G. A closed-circuit system that includes a Krogh-type spirometer for the continuous measurement of oxygen consumption was used to determine heat production. At the same time, core temperature (T_c) and tail temperature (T_t) were also measured. During the first 20 min. at 3 G, oxygen consumption increased by at most 18% in some of the rats and fell as much as 15% in the remaining rats. However, in all rats at 3 G, there were falls in T_t and T_c during this time. Thus, the initial fall in T_c at 3 G was independent of concurrent changes in the rate of oxygen consumption. Furthermore, the impaired mechanism responsible for the rapid fall in T_c during the initial exposure to hypergravity was not reduced heat production (since oxygen consumption was variable and in some rats increased) but an increase in heat loss. However, even after 3 hr. at 3 G, when heat conservation mechanisms had recovered, the rate of oxygen consumption in rats was not significantly increased relative to the 1 G rate; hence thermogenesis was not activated to rewarm the animal.

Second, data were also obtained on rats acclimated to a 2.1 G field and then cold exposed while in a hypergravic field of the same magnitude (2.1 G). Data obtained at this stage clearly indicate that acclimation to hypergravic fields enhances the ability of a rat to cope with cold exposure during centrifugation. In this experiment, 9th generation rats born and reared at 2.1 G were cold exposed at 2.1 G, and their rates of oxygen consumption were compared with those of rats born and reared at 1 G. This study is being performed in collaboration with Dr. Jiro Oyama at Ames Research Center.

Third, experiments on the resetting of the set-point for shivering and nonshivering thermogenesis are also in progress. The experiments are designed to test the proposal that in hypergravic fields, rats regulate their core temperature, but at

an abnormally low level. Preliminary data indicate that while core temperature is regulated, this regulation is complex in depending not only on the hypergravic field but also on the ambient temperature.

All three of the above studies center on the mechanisms underlying impaired temperature regulation in mammals exposed to hypergravic fields. This information is crucial to an assessment of this mammalian neural control system and provides background data for future studies in a hypogravic environment.

In addition to the neural processing of thermal signals, studies have been initiated on processing brainstem vestibular signals in the rat. Electrical circuits and associated mechanical elements to provide a precise vestibular stimulus to rats have been constructed and tested. In addition, the software for averaging individual vestibular responses has been developed. In preliminary experiments, vestibular evoked responses were recorded from rats using the vestibular stimulator and a laboratory computer.

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GRAVITY PERTURBATION AS A PROBE FOR ANALYZING PATTERN SPECIFICATION IN EARLY AMPHIBIAN EMBRYOGENESIS

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Gravity perturbation is being employed as a probe to study the manner in which early pattern (e.g., animal/vegetal polarity; dorsal/ventral polarity; primary axis morphogenesis) is specified. The eggs of most common laboratory amphibia (e.g., Xenopus, Rana, Ambystoma) display a prompt and immediate response to gravity, following activation (fertilization). Within minutes after sperm penetration, the typical amphibian egg rotates from its random orientation vis-a-vis gravity so that the darkly pigment animal hemisphere opposes gravity. Under natural conditions, amphibian eggs develop through the early pattern specification stages in that same orientation (animal hemisphere "up").

In order to employ gravity perturbation as a probe to understand the significance to pattern specification of egg rotation, a series of detailed analyses on morphogenesis in inverted Xenopus eggs, was carried out. Specifically, the cytoplasmic rearrangements which follow fertilization in both naturally oriented and inverted eggs were monitored. A set of yolk compartments was resolved by cytological analyses. Those compartments were characterized by their yolk platelet compositions and the manner in which they moved during egg inversion. During egg inversion several (but not all) of the yolk compartments shift. Those observations were coupled with additional observations on the pattern of morphogenesis in inverted eggs: The relocation of the major yolk compartments, but not some of the minor ones, is a prerequisite for normal pattern specification. Those findings were interpreted in terms of a novel "density compartment model" as a coherent way to view the organization of the egg cytoplasm and the specification of early pattern.

As an additional subcellular marker, the germ plasm (cytoplasmic localization involved in primordial germ cell (PGC) specification) was monitored in inverted eggs. Inverted eggs typically contain reduced numbers of PGCs. Although most of the major cytoplasmic compartments shift in inverted eggs, the germ plasm does not. Reduced PGC numbers might be due to the inability of the germ plasm compartment to adjust (by movement) to the novel gravity orientation of egg inversion.

As a further attempt to employ gravity as a probe for analyzing early pattern specification, Xenopus eggs were clinostatted. The

constant rotation of the clinostat serves to "cancel" the effects of natural gravity orientation. Several variations of previous clinostat protocols were employed, including fertilizing eggs directly on the clinostat (while rotating), and beginning the rotation after eggs were oriented in regard to the sperm entrance site.

Pattern specifications were monitored up to the tadpole stage. Briefly, although the normal orientation of the dorsal lip 180° opposite the sperm entrance site was not observed, morphogenesis was normal. Hence, it is predicted that the major pattern specification events of early morphogenesis will be normal in the microgravity of outer space.

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STRUCTURAL DEVELOPMENT AND GRAVITY

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During 1983, research concentrated on two major objectives. In addition to research goals, the laboratory was involved with the Weber Student Shuttle Involvement Project (SSIP) which was flown aboard STS-41B in February 1984.

The first major research objective was to define changes in bone formation rates and bone structure with age in the rat. Characterization of bone structure with age is essential since each bone appears to have a rate of formation and elongation and shape appropriate for its particular function and age. Two strains of rats were studied: (1) Fischer 344 and (2) Sprague-Dawley derived; these strains should obtain significantly different adult body size with the Fischer strain being smaller than the Sprague-Dawley. Total adult bone mass should be directly related to the size of the animal. This study was initiated in 1982 and was completed in August 1983. Rats were studied at 6, 10, 18, 36, and 68 wk. of age. Bone formation in the distal shaft of the leg bone (tibia-fibula junction) was most rapid at 6 wk. of age and precipitously decreased until between 18-36 wk. of age when bone formation appeared to plateau. The bone formation rate curve for both strains was very similar although the initial rate of bone formation was higher in the Sprague-Dawley animals and, hence, this strain had slightly larger bones. This curve was also essentially superimposeable on the curve reported by Pace and co-workers for basal metabolic rat vs. age suggesting that skeletal maturation and metabolic maturation occur simultaneously. Data from bone elongation and other bone sites are in progress.

The second research objective was to validate and refine the rat model used to simulate some of the physiological effects of spaceflight. Animals on the model for 1, 2, or 3 wk. were compared with animals exposed to 4^{o} C for the same period of time to determine whether endocrine stress (elevated glucocorticoid levels) might be contributing to the changes in muscle mass or bone mass produced by unloading the hindquarters of the rats on the model. Only animals exposed to the cold responded with an increase in adrenal weight and a decrease in thymus weight, data indicative of increased glucocorticoid activity. Only animals on the model showed true atrophy (actual loss of muscle mass) of the soleus muscle (an antigravity leg muscle) although the cold-stressed rats showed decreased muscle growth. Bone formation rate was decreased similarly during the first week in both groups, but only the cold-stressed animals tended to return

to normal rates during the subsequent times. These data demonstrate that animals on the model for 1-3 wk. are minimally stressed as they do not show alterations in glucocorticoid sensitive tissues as do cold-stressed rats. Thus, elevated glucocorticoids play only a minor, if any, role in the physiological responses of unweighting rat hindquarters; the major factor influencing such changes is unloading the hindquarters and/or fluid shifts induced by the head-down position of animals on the model.

The laboratory assisted with the Weber SSIP which was to determine whether the pathogenesis of arthritis involves a gravity component. The hardware test of the animal enclosure module (AEM) for housing the rats in the middeck of the shuttle was conducted aboard STS-8 which flew August 30-September 4, 1983. Six, healthy, male, Lewis gnotobiotic rats were included in this test which was managed by NASA-Johnson Space Center with the assistance of NASA-Ames Research Center. Following the successful hardware test, the experiment was scheduled for STS-41B. Dr. Holton was appointed NASA scientist/project manager for the Weber SSIP and established a biweekly newsletter for the project beginning in October 1983. The outcome of this project will be detailed in next year's progress report.

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REGULATORY MECHANISM OF SKELETAL MUSCLE ATROPHY AND FLUID AND ELECTROLYTE SHIFTS IN THE HYPOKINETIC/HYPODYNAMIC AND ANTIORTHOSTATIC RAT

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The focus of this research is the development and utilization of a dual purpose animal model to effectively plan experiments for weightlessness in orbital flight. Two areas are under investigation: (1) differential muscle atrophy resulting from nonloading suspension and the processes of recovery, using hind limb muscles (soleus, gastrocnemius, plantaris, and extensor digitorum longus (EDL)) in hypokinetic/hypodynamic (H/H) suspended rats; and (2) relationships between fluid and electrolyte imbalance and cardiovascular response in head-down suspended rats.

These studies extend from previous results of differential muscle atrophy (soleus > gastrocnemius = plantaris > EDL) from this lab which served to illustrate that the H/H rat model mimics and compares favorably with the results of rats exposed to weightlessness in the COSMOS series of experiments. The objectives of the muscle projects are currently focused on examination of contractile function and the endurance (i.e., the rate and extent of muscle fatigue) of hind limb muscles from H/H rats at various work intensities. In addition, several indices of metabolic activity before and after contractile activity are also measured (namely, glycogen content, lactate production, and oxidative capacity).

Examination of the endurance capacity of muscles from one-wk. suspended H/H rats showed that the atrophied gastrocnemius fatigues significantly faster than the soleus. The loss of endurance capacity in the gastrocnemius, but not the soleus, of H/H rats suggests a differential involvement of fiber types. Evolution of the limb musculature in relation to functional adaptation is readily illustrated in the soleus, which is predominantly a slow twitch, highly oxidative muscle, in comparison with the gastrocnemius which is a mixture of fast-twitch oxidative glycolytic and fast twitch glycolytic fibers. In addition, this raises a most interesting question, namely, despite the greater loss of soleus muscle mass the remaining nonatrophied soleus fibers are highly functional. In contrast, atrophy in the gastrocnemius, a mixed fiber muscle,

responds with a loss of some endurance capacity. Another series of experiments showed that cytochrome c concentrations (index of oxidative capacity) as well as activity of citrate synthase were decreased in gastrocnemius during one-wk. H/H suspension. Thus, the increased fatigability of the gastrocnemius may be due to a selective loss of the sarcoplasmic protein fraction. These and other speculative aspects will continue to receive attention in the 1984-1985 phase of this program.

The general conclusion to be drawn from these investigations is that despite muscle atrophy with short periods (1 or 2 wk.) of nonloading H/H, the muscle remains functional, provided that the work intensity is kept low. Future experiments will test this hypothesis by doubling the work intensity. In addition, recovery tests will be aimed at discovering the time it takes for full recovery of endurance capabilities. Since muscle fiber type has been found to be related to the fatigue reactions, an analysis of specific fiber type alterations in response to atrophy will be done using image analysis, histochemistry, and electron microscopy techniques.

The second area of research, involving previously demonstrated disturbances in fluid and electrolyte excretion will be examined in terms of correlated cardiovascular responses. The basic hypothesis has been that animals in the model, that is, head down, whole body suspended rats, respond with a cephalad fluid shift. This fluid shift, in response to an altered gravitational orientation, is one of the chief characteristics of orbital flight. Since no direct experiments have been performed with experimental animals under conditions of weightlessness, the only relatable studies are those with the human subject. In the astronaut exposed to weightlessness, for varying periods, there is a recognized excess output of urine and electrolytes. The same conditions were obtained in water-immersed animals and humans, and in prolonged bed-rested humans. Since the animal model, the bed-rested human and the weightless human can be said to experience unusual gravitational vector orientation, there is a working model for future understanding of the disruption of the normal gravitational vector in the mammal.

Two areas of investigation are underway. In one series of experiments, vascular pressure measurements showed increased blood pressures (mean, systolic, and diastolic) in head-down tilted rats. This could, in general terms, be characterized as a hypertensive response. Such a response is also characteristic of an increased atrial blood volume and suggests a response to baroreceptor reflexes. Some preliminary studies have been published.

A second series of studies involves the role of hormone regulators, namely, aldosterone (regulating sodium retention) and antidiuretic hormone (regulating water retention). It is

proposed that in the antiorthostatic rat, that is, the head-down tilted subject, these hormones may play an important role in the genesis of the fluid and electrolyte disturbances (e.g., increased excretion of urine and electrolytes). Similar conditions have been demonstrated in human studies during spaceflight and now can be simulated with the animal model. It has been hypothesized that the level of both hormones should be depressed to account for the diuretic and natriuretic effects. Preliminary results, however, have demonstrated an early (after 1-3 days of suspension) increase in plasma aldosterone concentration, suggesting that the fluid and electrolyte disturbance may result from a complex series of hormonal events. Detailed studies for 7- and 14-day subjects are underway and will continue in the 1984-1985 phases of this program.

In summary, the animal model used in this laboratory has proven valuable for investigating disturbances in basic muscle physiology and in fluid and electrolyte regulation. Future studies are intended to further elucidate specific mechanisms in order to plan crucial flight experiments and to determine the mechanisms involved in the course of recovery phenomena.

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HYPER-GRAVITATIONAL EFFECTS ON METABOLISM AND THERMOREGULATION

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The objective of the research program is to develop a more comprehensive and basic understanding of the effects and mechanisms of action of acute and long duration hypergravity exposures on mammalian organisms. Investigations are focused on systems regulating blood glucose and body temperature of the rat.

Studies during 1983 have been aimed at deterimining: (1) the effects of acute hyper-G stress on gluconeogenesis and glucose homeostasis, (2) basal metabolic rates of hyper-G-adapted rats, and (3) the relationship between G intensity and the hypothermic response induced by hyper-G stress exposures.

Results from these studies have further delineated the important role of the sympathetic-adrenal system on the initial rapid rise in blood glucose during hyper-G stress exposures. Results from gluconeogenic rate measurements using labeled lactate, alanine, and glycerol in hyper-G-stressed rats have shown that the initial surge in blood glucose is due mainly to an increase in gluconeogenesis from lactate (from catecholamine-induced muscle glycogenolysis). Since the initial increase in plasma catecholamine levels is only transient returning to control levels after 1 hr. of hyper-G exposure, the sustained increase in gluconeogenesis and in blood glucose levels with longer exposure durations appears to be mediated by glucagon action. The increased plasma glucagon levels found correlate well with the increased gluconeogenic rates and the increase in blood glucose levels found in hyper-G-stressed rats.

A considerable amount of effort was expended during 1983 to gain experience with and to improve upon the previously developed procedures to isolate hepatocytes from livers of hyper-G-stressed rats for in vitro gluconeogenic rates measurements. Problems affecting the viability of the hepatocytes as well as yields were finally resolved, and gluconeogenic rate measurements using labeled lactate and alanine were initiated on livers from hyper-G-stressed (0.5 hr.; 3.1 G) and control rats. The method used differs from all other published methods in that cannulation and perfusion are performed immediately after decapitating the rat thereby obviating any possible effects of anesthetic drug actions on the rate measurements.

Data obtained over a number of years on oxygen consumption rates of different groups of hyper-G-adapted and normal gravity control rats have been analyzed along with data on body composition. Results of the analysis, which takes into account differences in

body mass, body composition, and age, provides additional evidence that the resting metabolic rates of hyper-G-adapted rats are significantly higher (as much as 30%) than comparable rats reared under normal gravity. A paper on this study will be submitted for publication shortly.

An extensive series of acute hyper-G exposure studies were completed in which the effects of centrifuge radius, rotational rate, and G intensity on rectal and tail temperature responses of rats were determined. The important finding was that the changes observed in rectal temperature (decrease) and tail (increase) are directly and solely related to the G intensity imposed on the animal and are independent of the radius and rotational rates used. Identical temperature responses were obtained for the same G intensity when the radius ranged between 0.46-1.73 m and the rotational rate ranged between 22-44 RPM.

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GRAVITY, BODY MASS AND COMPOSITION, AND METABOLIC RATE

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The purpose of this project is to examine energy metabolism and body composition characteristics of mammals as a function of body size in order to assess the evolutionary influence of gravitational loading on present-day scaling relationships. Five species of common laboratory mammals--the mouse, hamster, rat, guinea pig, and rabbit--have been selected for examination. This series provides a 100-fold range of mature body mass from 0.05 to 5 kg.

Six male and six female animals of each of eight age cohorts from 1 mo. to 24 mo. for each of the five species form the basic animal matrix of the study. The oxygen consumption rate of each animal is measured under standard conditions to provide metabolic rate, and the animal is then killed for organ mass measurements and biochemical analysis for body contents of water, fat, potassium, sodium, magnesium, calcium, phosphorus, nitrogen, and creatine. From these parameters it is also possible to derive fat-free body mass, body cell mass, intracellular and extracellular water masses, bone mineral mass, and skeletal muscle mass. Quantitative scale relationships of the parameters to body mass are determined by allometric analysis. Comparison of the data among the various age groups also permits separation of growth effects from loading effects.

By the end of 1983 the data for the animal matrix was about 90% completed. All the metabolic rate, body water content and body fat content measurements were in hand, and the biochemical analyses of the dried, fat-free, whole body powders for all but two age cohorts were done. Although definitive treatment of the data awaits completion of all the analyses, several preliminary findings have emerged. For example, the three major body components--skin, viscera, and skinned, eviscerated carcass--have been considered separately. The skinned, eviscerated carcass comprises all of the body skeleton and practically all of the skeletal muscle, and may be regarded as the weight-bearing organ of the body. It has been shown that for the series, mouse to rabbit, the allometric relationship between carcass mass and total body mass (TBM) is:

$$\text{Carcass: kg} = 0.503(\text{TBM, kg})^{1.055};\ r = 0.999.$$

Whereas, the relationship for viscera mass to TBM is

$$\text{Viscera: kg} = 0.158(\text{TBM, kg})^{0.871};\ r = 0.995,$$

and for skin it is

$$\text{Skin: kg} = 0.139(\text{TBM, kg})^{0.942};\ r = 0.993.$$

Thus, Galileo's classic theory that the scale of the weight-bearing components of the body must increase proportionately to body mass as animals become larger, and by implication the visceral components must diminish proportionately, has been confirmed. Allometric analysis of the relationships of the body elements to body mass will permit further definition of the influence of gravity on the evolution of body characteristics.

Another preliminary finding has been that when metabolic rate is expressed as metabolic intensity, that is, in the mass-specific terms of kcal$\cdot$ hr.$^{-1}\cdot$kg body mass^{-1}, and is examined as a function of age in this series the relationship is hyperbolic in form. Metabolic intensity is high in young animals, and falls asymptotically to a relatively constant value by age 5-8 mo. in these species. Thus, it is possible to define metabolic maturity quantitatively, and to demonstrate that the interspecific 3/4 power allometric scaling of metabolic rate on TBM holds only for metabolically mature mammals.

During 1983, study of the intracellular concentration of creatine in skeletal muscle tissue from the series of animal species has been extended. The development of a method for estimating skeletal muscle mass of mature animals 8 mo. or more in age from their body creatine content has been reported previously. Skeletal muscle tissue from 2-, 3-, and 5-mo.-old animals is now being examined with the objective of expanding the method to include these younger animals. It is anticipated that the results will make possible interpretation of the body creatine data from the basic animal matrix in terms of skeletal muscle mass as a function of species, sex, and age.

In other experiments, the effects of ambient temperature change and of increased gravitational loading by chronic centrifugation on the allometric relationship between metabolic rate and TBM in metabolically mature animals of the series have been examined. It is well known that the metabolic rate of mammals is at a minimum in a relatively narrow ambient temperature range known as the thermoneutral zone. When ambient temperature falls below, or rises above, the thermoneutral zone, the metabolic rate increases. The precise range of the thermoneutral zone is a function of species, age, and sex differences, and has been established only for a relatively few cases. Hence it was necessary for the thermoneutral zone range to be determined for the animals of this series. It has been found that $28 \pm 1^{\circ}$ C represents the ambient temperature range within which the metabolic rate remains within $\pm$ 10% of the minimum value for metabolically mature animals of the species used.

During 1983 it was shown that in this ambient temperature range the interspecific allometric relationship between metabolic rate and TBM for the series is given by

$$\text{Metabolic Rate, kcal}\cdot\text{hr.}^{-1} = 2.91(\text{TBM, kg})^{0.766};\quad r = 0.979,$$

in good agreement with the classic Kleiber 3/4 power relationship for mammals generally.

However, when the same animals were measured in an ambient temperature environment of 24 $\pm$ 1^{o} C, the allometric relationship became

$$\text{Metabolic Rate, kcal}\cdot\ \text{hr.}^{-1} = 3.51\ (\text{TBM, kg})^{0.676};\quad r = 0.987.$$

The positioning constant of the equation is elevated because of the higher metabolic rate at the lower temperature, and the exponent is lessened because the metabolic rate of the smaller animals was increased proportionately more at the lower temperature than was that of the larger animals. Hence, it may be concluded that the condition of thermoneutrality is important for experimental studies of the effect of altered gravitational loading on metabolic scale effects.

Such a study was completed during 1983 of the effect of 6 wk. of chronic centrifugation at 2.0 G on the allometric scale relationship between metabolic rate and TBM in metabolically mature animals of the species. The relationship was found to be

$$\text{Metabolic Rate}_{2.0G},\ \text{kcal}\cdot\text{hr.}^{-1} = 3.30(\text{TBM, kg})^{0.813};\quad r = 0.993.$$

This finding has led to the conclusion that augmented gravitational loading shifts the relationship between metabolic rate and TBM by an increase in both allometric parameters, as was predicted previously, and that therefore gravitational loading is an important contributor to mammalian metabolic energy requirements. A continuing prediction is that abatement of gravitational loading in spaceflight will result in a lowering of both allometric parameters in the classic 1.0 G 3/4 power relationship between metabolic rate and TBM.

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EFFECT OF DECREASED GRAVITY ON CIRCULATION IN THE RAT

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Limiting movement, or hypokinesia, in the antiorthostatic (head-down) position mimics the circulatory effects of weightlessness. Circulatory processes in the rats were studied during their exposure to head-down (-20^{o}) hypokinesia, and during readaptation of the rats to control conditions. The cardiovascular measurements were done in control experiments on unrestrained, unanesthetized rats, and in the same animals in hypokinetic conditions, and during readaptation to free activity. Because surgery and anesthesia drastically decrease cardiac output and other circulatory parameters in rats, measurements were taken from unanesthetized rats. For sampling blood during the study, the aortas and right ventricles of the animals were permanently cannulated 10-15 days before the experiment.

Investigators measured the hormones ACTH, corticosterone, and prolactin by radioimmunoassay to monitor the stress imposed on the rats by placement of the suspension harness and by the head-down position. These stress hormones rose in the early exposure to antiorthostatic hypokinesia, but after 6-7 days of exposure the animals adapted to the unusual position, as judged on the return of these three hormones to their normal levels. Plasma catecholamines were also elevated on days 1 and 3 of the antiorthostatic exposure. The plasma growth hormone level was decreased on day 1. On day 7 of the exposure, the plasma levels of both hormones were only slightly above the control levels. After release from the harness and after return to their own cages, the hormone levels were again elevated, although not as much as during the antiorthostatic exposure. The elevation lasted 2-3 days. In harnessed animals that were orthostatic (not positioned head-down), the increase in the plasma level of stress hormones was very small and lasted only a few hours on the first day. The level of hormones was at or near control values during the next 6 days. Harness removal did not induce any increase of studied hormones.

Investigators studied hormonal stress levels during 30-min. immobilization in rats, repeated 17 times during a 17-day period. Adaptation processes were completely absent during repeated immobilization, while during exposure to antiorthostatic hypokinesia, the animals adapted after 3-4 days, and in the case of orthostatic hypokinesia this process was only a few hours long.

In other studies the ADH levels decreased the first day of exposure to antiorthostatic hypokinesia, but angiotension II levels were only slightly elevated. Also, cardiac output in antiorthostatic rats followed circadian patterns 4-7 days after initiation of hypokinesia.

BONE CELL KINETICS OF SIMULATED WEIGHTLESSNESS

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In general, this project addresses the influence of gravity on mammalian cell differentiation (specialization). Specifically, the focus is on the mechanism by which production of osteoblasts (bone-forming cells) is inhibited in weightlessness. Osteoblast differentiation is studied in weight-bearing long bones (tibia and ulna) as well as in maxillary periodontal ligament, adjacent to the roots of teeth in the non-weight-bearing upper jaw. The cytological marker for distinguishing preosteoblasts (the immediate predecessors of osteoblasts) from less differentiated and nonosteogenic cells is nuclear size. Differentiation of a committed osteoprogenitor cell (A') to preosteoblasts (C/D cells) involves a 200-300% increase in nuclear volume. This increase in nuclear size is a morphological manifestation of change in differentiation.

During 1983, focus was on the following objectives: (1) define the proliferation and differentiation sequence for osteoblast production (histogenesis); (2) determine the effect of simulated weightlessness, via head-down suspension of a rat by its tail, on osteoblast histogenesis in the periodontal ligament (PDL); (3) develop an autoradiographic technique (histological detection of radioactive tracers) for distinguishing between ^{14}C and ^{3}H labels in the same specimen; (4) utilize the nuclear morphometric assay for osteoblast precursors to assess the effects of 18.5 days of weightlessness on the ulna of Cosmos 1129 rats; and (5) in a related project with pilot funds from another source, develop a method of using rigid, endosseous implants to test osseous adaptation to a defined interval load, in bone of a true remodeling species (rabbits).

Progress

1. Osteoblast Histogenesis Sequence

Extensive analysis of previously published and unpublished cell kinetic data has resulted in identification of a five-compartment model for osteoblast histogenesis (proliferation and differentiation sequence for forming a specialized cell): (1) self-perpetuating, relatively undifferentiated precursor cells (A); (2) committed osteoprogenitor cells (A'); (3) G_1 stage (pre-DNA synthesis) preosteoblasts (C cells); (4) G_2 stage (post-DNA synthesis) preosteoblasts (D cells); (5) mature osteoblasts. Nuclear volumes are 40-80 μm^3, for A and A' cells,

120-169 μm^3 for C cells, and $\geq$ 170 μm^3 for D cells. Mathematical simulation of osteoblast production, based on the numbers of each cell type present in the rat PDL, has resulted in a close approximation of two sets of experimental data. This suggests the present model of osteoblast differentiation has identified all major osteogenic components among fibroblastlike cells of the PDL. As far as is known, this is the first osteoblast differentiation sequence which has been worked out in any bone-forming tissue.

2. Weightlessness vs. Simulated Weightlessness

Both weightlessness (COSMOS) and simulated weightlessness (30° head-down suspension by the tail) results in significant decreases in the numbers of preosteoblasts (C/D cells). This suggests an inhibition of the key rate limiting step in osteoblast histogenesis, which is the increase in nuclear size (A' → C) to form a preosteoblast. Since both weightlessness and simulated weightlessness have a similar net effect, of decreasing preosteoblast production, the block in the A' → C shift is not a direct effect of weightlessness. It may be related to a generalized disturbance in cell physiology, associated with the well-documented fluid shift and loss of extracellular water.

3. $^{3}H/^{14}C$ Tracer Studies

A low dose (0.1 μCi/gm) of ^{14}C-thymidine (DNA precursor) and a higher dose (3.0 μCi/gm) of ^{3}H-lysine, successfully distinguished between labeled nuclei (DNA synthesis) and labeled cytoplasm (protein synthesis). Routine autoradiography with 7-10 days of exposure produces a good assessment of protein synthesis, which is not significantly contaminated by the low dose of ^{14}C which is restricted largely to the nucleus. A special technique for detecting the ^{14}C labeled nuclei was developed. Histological sections receive a 3-5 μm coat of gelatin prior to dipping in nuclear tract (autoradiographic) emulsion. Since ^{3}H has a disintegration range of only about 1.0 μm it is not registered in the emulsion. Exposure of 3 mo. is necessary to record ^{14}C-labeled nuclei because of the low injected dose.

The only major problem is that ^{14}C-labeled nuclei cannot be distinguished from unlabeled nuclei in a dense population of cells, like bone marrow. The energy of ^{14}C is sufficiently high that a halo of disintegrations is registered for 4-5 μm around each labeled nucleus. This problem would significantly interfere with analysis of some populations of bone-lining cells. In conclusion, it is best to use separate animals to assess DNA and protein synthesis, but it is possible to simultaneously assess both in the same animal using the present double label technique, at least in areas of relatively low cell density. This method presents an option for maximizing the scientific yield from a small number of animals.

4. Osteoblast Differentiation of Long Bones

Nuclear size analysis of fibroblastlike cells in the primary spongiosa (just beneath the growth cartilage) of rat ulna specimens from Cosmos 1129 (18.5 days of spaceflight) show a similar net depression of the number of C/D cells as PDL. These results indicate: (1) the osteoblast differentiation sequence developed in PDL is applicable to other skeletal sites, and 2) osteoblast histogenesis is inhibited in the growing long bones in weightlessness.

5. Osseous Adaptation in Rabbits

A titanium endosseous implant has been devised which develops a rigid, osseous interface with cortical bone. A spring delivering 100 gm of compression is applied 6 wk. after implants are placed. Periosteal areas exposed to concave flexure display osseous hypertrophy to compensate for the load. This is a well-defined means of assessing adaptation to a known load. The response can be assessed with radiographs and multiple colors of bone-staining dyes which mark areas of bone formation at the time of injection. This method could be utilized to test skeletal adaptation to applied loads (form/function interaction) under conditions of weightlessness and simulated weightlessness. It is readily adaptable to quantitative procedures such as histomorphometry (measurement of bone turnover using vital labels) and nuclear morphometric assessment of osteoblast histogenesis.

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MAMMALIAN GRAVITY RECEPTORS: STRUCTURE AND METABOLISM

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The long-term goal of this research is to elucidate calcium metabolism in mammalian gravity receptors. To accomplish this objective it is necessary to study both the mineral repositories of the receptors, the otoconia, and the sensory areas themselves, the saccular and utricular maculas. The main focus of this research project has been on the otoconia.

Mammalian otoconia (literally "ear dust") are minute particles of calcite that mimic the form and symmetry of naturally occurring single crystals. Otoconia contain organic material in addition to calcite, and organic material also binds the particles to one another and to the underlying macula. The organic material below the otoconia has, in particular, been referred to as the "otoconial (or otolithic) membrane." It is commonly accepted that otoconia simply increase the mass of the organic membrane to magnify the inertial force acting upon the receptors (hair cells) under conditions of linear acceleration (or deceleration). Early results of this study demonstrated, however, that otoconia are dynamic mineral deposits and suggested that they function in calcium regulation in the system. The rate of uptake and release of radioactive calcium in vivo and in vitro strongly indicated that organically directed mechanisms, rather than processes involved in inorganic crystal growth, were responsible.

These early findings led to the investigation of the role of organic material in facilitating the seeding and growth of the mineral deposits and in controlling their form and mass. Fetal studies in the rat indicated that deposition of organic and inorganic otoconial phases was nearly simultaneous developmentally. The two phases appeared identical in organization within the crystals. These results prompted more recent explorations of: (1) the structural organization of otoconia and related otoliths, (2) the evolution of the mineral deposits in vertebrates, and (3) the precise chemical composition of the organic otoconial phase in various vertebrate species. This research is being carried out through the use of ultrahigh resolution transmission electron microscopy to determine the lattice structure of the mineral phase; scanning electron microscopy of otoconia of species representative of the various vetebrate orders; and high performance liquid chromatography of the amino acid and carbohydrate composition of the organic material of inner ear mineral deposits.

A comparative, ultrahigh resolution transmission electron

microscopic study of fish otolith, frog otoconia, and rat otoconia was reported during 1983. This study showed that none of the otoconia investigated were single crystals nor were the otoliths polycrystalline, as had been suggested by previous crystallographic research. All were composites of highly ordered crystallites. In the case of rat otoconia, the crystallites were typically ~80 nm in broadest diameter and had some curved and some sharp edges. In contrast, naturally occurring calcite (Iceland spar) broke into fragments of nonuniform sizes having sharp edges. Electron beam diffraction patterns obtained from all the inner ear mineral deposits showed numerous imperfections in comparison to their inorganic counterparts. The results taken together were interpreted to indicate that organic material was important in the seeding and growth of the unusual crystallites included in an otoconium or otolith, and for ordering them into the final form. An important, coincidental conclusion based upon crystallite asymmetry was that otoconia might function piezoelectrically, that is, by altering the electric field around the hair cells rather than by mechanical bending of the hair bundles of the receptor cells.

Another observation was that the diffraction patterns sometimes obtained from fragmented frog otoconia, which typically contain aragonite rather than calcite, matched the hexagonal patterns yielded by some rat otoconial fragments. The spacings in hexagonal aragonite and calcite diffraction patterns (001 faces) are nearly identical and make it impossible to distinguish between the two polymorphs of calcium carbonate on these grounds alone. Nevertheless, the observation raised the questions whether calcitic otoconia were present in the frog, or whether the calcitic configuration evolved from a prior, aragonitic form which it mimicked.

The questions raised led to more recent, scanning electron microscopy of otoconia from a number of species representative of the various vertebrate classes. In June 1983 it was reported that only calcitic-type otoconia were present in turtle utricles. In the American alligator, calcitic-type otoconia occurred in all its gravity receptors. These reptiles were studied because they descended from the same vertebrates that served as ancestors for mammals and for birds. Occasional calcitic-type otoconia in amphibian gravity receptors, have also been observed but the study is incomplete.

The scanning electron microscope results clearly showed that the calcitic configuration preceded the evolution of birds and mammals, but it has not yet been demonstrated whether the mineral in such otoconia is calcite or aragonite. The findings led to deeper questions: Precisely what determimes whether aragonite or calcite is to be deposited in a particular gravity receptor? What difference, if any, does this make to the functioning system? The chemical composition of the organic material and the number

of proteins involved needs to be known to learn the answers to these questions. Although this line of research began several years ago, using microdisc gel electrophoresis methods, high performance liquid chromatography (HPLC) and a sensitive fluorescence detector have been used more recently to analyze otoconial masses for their amino acid and carbohydrate constituents.

The first analysis of the amino acid composition of otoconia was presented during the summer of 1983 at an international meeting on biomineralization. The findings were that otoconial complexes (otoconia plus otoconial membrane) were high in acidic and low in basic amino acids. The original method employed for amino acid analysis involved postcolumn derivitization with a substance called O-phthaldialdehyde (OPA). This method did not permit detection of proline and hydroxyproline, which are significant components in collagen. (Collagen is important in biomineralization of bone.) In late 1983, therefore, analyses using hypochlorite (clorox bleach) in addition to OPA were carried out to determine whether proline and hydroxyproline were important constituents of otoconial organic material. The results, reported in February 1984, were that small amounts of these two amino acids occur in otoconial matrix, but they are relatively insignificant.

In collaborating with a colleague in the Department of Pharmacology, Dr. Fulvio Perini, otoconial organic material was analyzed for the presence of amino and neutral sugars. It was demonstrated that otoconial organic material contains relatively large amounts of glucosamine and galactosamine as well as galactose, and smaller quantities of mannose. The findings could indicate that presence of glycoproteins containing N-linked oligosaccharides, possibly with a repeating galactose-N-acetylglucosamine sequence, as well as O-linked oligosaccharides of the type sialic acid-galactose-N-acetylglucosamine.

The results of the HPLC analyses are exciting. They show that otoconial complexes, like other biomineralized materials (such as shells and otoliths) that contain a polymorph of calcium carbonate, have organic material that is high in acidic and low in basic amino acids. Moreover, the carbohydrate findings indicate that the organic matrix is, or contains, glycoprotein(s). In many other biomineralizing systems, it is a soluble, highly sulfated acidic glycoprotein that is considered to be important in the seeding and growth of crystallites, and in inhibiting crystallite growth beyond a certain size. That is, the acidic, aspartic acid residues of the glycoprotein attract calcium, then carbonate ions to seed a crystallite by ionotropy. The crystallite grows to a certain size whereupon acidic glycoprotein in solution in the surrounding medium is adsorbed, stopping further growth of that small crystal. Free acidic, aspartic acid residues of the adsorbed glycoprotein are, however,

able to attract calcium ions to repeat the process, enabling the biomineralized material to grow in total size. While not all aspects of otoconial growth and inhibition are thus explained, the hypothesis would fit the nearly simultaneous deposition of organic and inorganic materials observed in developing otoconia.

Muriel Ross gave a paper on her new findings concerning the complexity of innervative patterns in rat gravity receptors at the American Medical Association Meeting in San Diego in May 1984. Her ultrastructural results indicate that the two kinds of receptor cells in the maculas are integrated functionally through shared afferents and that there exists a system of efferent type nerve fibers and terminals of intramacular (mostly calyces) origin. Additionally, there is a plexus-like arrangement of afferent and efferent-type terminals at many sites in both maculas. On the basis of her finding, Dr. Ross suggested that sensory processing of information concerning linear acceleration begins peripherally. The finding of an intramacularly originating system of efferents may provide anatomical evidence for lateral inhibition in the macula, to improve signal to noise, and for peripheral adaptation to constant acceleration. Because of the complexity of the neural patterns both on the afferent and the efferent neural side, Dr. Ross hypothesized that naturally occurring asymmetry in macular neural organization may be common, and that variability in degree of asymmetry may provide a peripheral contribution to the expression of space adaptation syndrome in the novel environment of microgravity. The results provide an anatomical basis for complex sensory processing in the maculas, and suggest that macular asymmetry may be common.

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SOME ASPECTS OF HYPOGRAVITY SIMULATION ON BONE FORMATION AND MATURATION

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Research is directed toward an understanding of the use of land-based hypokinetic systems (LBHS) to model the effects of hypogravity on the skeleton. The particular focus has been to determine the applicability of LBHS on the non-weight-bearing skeletal parts which do not show the severe osteopenic consequences of spaceflight and unloading observed in the weight-bearing bones. However, the non-weight-bearing bones are not unaffected by spaceflight. The jaws and teeth of rats flown in the Cosmos-1129 Biosatellite showed a defect in the rate of maturation of the mineral and matrix moieties, indicating that the changes in these bones might discriminate between specific effects of unloading and specific effects of gravity.

This report deals with three experimental land-based hypokinetic systems--a rat suspension model and two primate restraint systems. In the suspension model, the limb bones are partially unloaded by raising the hindquarters of rats at a 30^{o}-40^{o} angle (NASA-Ames Research Center). The primate restraint systems involve postcranial whole body plaster casts, or chair-restraint on a roto-positioner (Wright Patterson AFB). All systems, then, mimic the effects of skeletal unloading on the weight-bearing bones, but none of them should have any special influence on skeletal parts such as the jaws which are already "unloaded" save for the stresses placed on the bones by antigravity muscles. Thus, where the jaws are concerned, these systems then test more specifically for an effect of gravity.

Several techniques were applied to this work. First, tetracycline labeling was used so that the rates of appositional bone growth could be followed accurately during the periods of immobilization and postimmobilization recovery. Second, a bromoform-toluene density gradient fractionation method was used to follow the rate of maturation of newly forming mineral (calcium and phosphorus) and matrix (hydroxyproline) moieties. It was the latter method which defined the failure of maturation in the jaws of rats actually flown in space.

Rat Suspension Model: The most striking result from this series of studies was the complete absence of abnormal patterns of appositional bone growth (i.e., mineralization rates) and tissue maturation in the jaws of rats entered into the suspension model. Because this model did not mimic the effect of spaceflight, there

is reason to believe that at least some of the processes involved in tissue maturation have an important gravity component.

Primate Immobilization Model: During postcranial immobilization (plaster casting), the freely moving jaws of immature 4-6 kg Rhesus monkeys exhibited normal rates of lamellar (periosteal and endosteal) bone formation. However, a population of osteons showed a failure of normal appositional growth (tetracycline labeling), and the more trabecular regions of the jaw exhibited abnormal patterns (sp.gr. distribution) of mineral and matrix maturation. The effect on osteon growth and trabecular bone maturation was not observed in older 6-10 kg monkeys. Therefore, this model of hypokinesia is most sensible to the youthful, rapidly growing skeleton.

Primate Immobilization (Roto-positioning) Model: In this system, 9 juvenile 4 kg monkeys were restrained in a supine position and rotated (using the Primate Rotational System, Patent No. 4120266) 90^{o} every 30 min. through a full 360^{o} arc for 14 days (RP/14). These animals maintained normal growth. Six were allowed to recover unrestrained in metabolism cages and were killed after 28 days (RP/14+28) and 56 days (RP/14+56). Three monkeys were maintained as controls (C) in metabolism cages. Osteon growth was normal in all groups, and roto-positioning did not effect changes in the porosity on the inferior mandible or change the normal frequency of tetracycline-labeled osteons with different microradiographic mineral densities. The patterns of mineral and matrix maturation have not yet been investigated.

The data to date do not suggest that it is feasible to mimic the effects of spaceflight on bone maturation in the jaw by imposing hind limb unloading hypokinesia at 1-G conditions. The data support the concept that bone growth and maturation have a gravity component.

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EFFECTS OF MUSCLE ATROPHY ON MOTOR CONTROL

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One of the goals of the Space Biology Program is to assess the physiological effects of gravity. Much of the animal data addressing this concern have been obtained from rats that were raised in small laboratory cages. The current objective of the research program is to determine the appropriateness of this small-cage rat as an animal model for the study of physiological adaptations to reduced-use conditions. Since the physiological status of muscle is traditionally quantitated by its speed of contraction, the maximum force it can exert, and its fatigability, these criteria were used to address two issues: (1) Given that the muscles of small-cage-reared rats are more fatigable than theoretically expected does transmission failure at the neuromuscular junction contribute to this impairment? (2) Does cage size during rearing affect the physiological status of selected rat muscles?

With regard to the first issue, the maximum force elicited by stimulation of the nerve was compared with that produced by stimulation of the muscle directly. Any difference between the two forces is typically interpreted as an impairment of transmission of the electrical signal across the neuromuscular junction. Although use of this paradigm has been reported in the literature for at least 35 yr., the inadequacy of this interpretation has not been well documented. In particular, when the muscle is stimulated directly it appears that the primary or initial site of initiation of the muscle action potential is the neuromuscular junction via the intramuscular branches of the nerve and not the sarcolemma as is generally supposed. In confirmation of this, experiments revealed no difference in the maximum force produced by stimulation of the nerve relative to the muscle. This does not, unfortunately, resolve the issue of neuromuscular junction-transmission failure.

In subsequent preliminary experiments, an attempt was made to compare the force exerted in response to nerve stimulation with that produced by stimulation of the muscle once it had been curarized; since curare blocks neuromuscular junction transmission, initiation of the action potential in response to direct-muscle stimulation in a curarized muscle cannot occur at the neuromuscular junction. Thus, any difference in force in these two conditions would reflect a neuromuscular-junction effect. Preliminary results indicate that direct-muscle stimulation occurs through the intramuscular branches of the axon and not through the sarcolemma. However, completion of these

experiments awaits the acquisition of some necessary equipment.

To address the cage-size issue, 30 Sprague-Dawley weanling male rats were randomly divided into two groups and raised for 98-155 days in either of two sized cages. One group of 15 was raised in the conventional small cages while the other group was reared in a cage that was 476X larger. For comparison with previous data in the literature, the physiological tests were performed on the soleus and extensor digitorum longus muscles. As an antigravity muscle, soleus is representative of muscles which serve both postural and movement functions and hence is thought to be more susceptible to the demands of usage. In contrast, extensor digitorum longus is less active and perhaps less adaptable.

Although the analyses are still in progress, preliminary results tend to support this generalization. Cage size did not affect either the weight of extensor digitorum longus or the maximum force it could exert. The soleus of large-cage animals, however, exhibited a doubling of the cross-sectional area of some of its muscle fibers and hence was heavier and stronger than the soleus of the small-cage rats. In addition, cage size had a substantial effect on the fatigability of both muscles; the muscles of the small-cage rats were about twice as fatigable. Of the three physiological criteria, therefore, preliminary data indicate that cage size influences the maximum force and the fatigability of muscle.

Given that these observations will be further substantiated as experiments continue, the next determination must be whether the adaptations of large-cage rats to reduced-use conditions (e.g., suspension hypokinesia) are comparable to those of small-cage animals. The outcome of this comparison will determine the appropriate animal model that will be used in subsequent experiments which explore the mechanisms associated with these adaptations.

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ADAPTATIONS OF RAT SKELETAL MUSCLE TO SUSPENSION-HYPOKINESIA

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A well-documented consequence of spaceflight is skeletal muscle atrophy. Research goals are to investigate the physiological changes associated with the influence of zero gravity on muscle function by first establishing a model for simulating zero gravity. Subsequent goals are (1) to investigate the underlying mechanisms responsible for the muscular changes caused by the model and by spaceflight and (2) to develop suitable conditioning regimens both to possibly preclude changes in muscular function for the purpose of facilitating the performance of man in space and to enhance recovery to normal muscle function upon returning from space.

Muscle atrophy has been investigated extensively using techniques that limit function to isometric contractions by limb immobilization and consequently, prevent passive limb movement, which is a characteristic of zero gravity. Fortunately, an alternative method for evaluating reduced muscular activity, was developed by Morey-Holton and permits unrestricted limb movement while reducing the loading influence of gravity on hind limb muscles. The Morey-Holton method suspends a rat so that its hind limbs are non-weight bearing.

The initial goal of the research was to determine whether the Morey-Holton technique simulates the influence of zero gravity on skeletal muscle. Although this validation procedure continues to be a part of current research, the data acquired indicate the Morey-Holton method is a valid model for weightlessness: (1) Atrophy of the soleus muscle and lack of atrophy of the gastrocnemius muscle were common consequences both of Cosmos 605, 690, and 782, and of rat suspension by the Morey-Holton method. (2) Both zero gravity and the Morey-Holton method evoke greater atrophy of slow-twitch fibers as compared with fast-twitch fibers in both the soleus and gastrocnemius muscles. This finding supports the accepted contention that slow-twitch fibers function to resist gravity or maintain posture while fast-twitch fibers are responsible for locomotion requiring greater force and velocity. (3) Soleus contractile function is reduced in both space-flown and Morey-Holton-suspended rats. (4) Although data are not conclusive, speeding of the soleus contraction suggested by Cosmos 605 and 609 is a significant result of rat suspension by the Morey-Holton technique.

In view of the resulting cited evidence of previous investi-

gations that the Morey-Holton technique provides a valid model for simulating zero gravity, the 1983 research became more involved with investigating the underlying mechanisms responsible for the muscular changes evoked by both spaceflight and the Morey-Holton method.

One such goal is to investigate the mechanisms involved in the greater atrophy of the slow-twitch fibers as compared with fast-twitch fibers. From cross sections of soleus muscles stained for myosin ATPase, the greater area occupied by all the slow-twitch fibers as compared with that of the fast-twitch fibers (slow-twitch fiber predominance) in control muscles was reversed by the suspension to a greater area occupied by fast-twitch fibers (fast-twitch fiber predominance). Additionally, measurement of individual fiber areas showed that slow-twitch fibers had a greater area in control soleus muscles, but that after 2 wk. of rat suspension, the fiber areas of the fast-twitch and slow-twitch fibers were equal. These data raise the possibilities that the change to fast-twitch fiber predominance with rat suspension could result either from greater atrophy of slow-twitch fibers and from conversion of slow-twitch to fast-twitch fibers. Research in 1984 is attempting to discern between these two possibilities by counting the number of fibers in the soleus muscle in control and suspended rats.

Although the speeding of the soleus contraction with rat suspension and spaceflight can partially be explained by the observed change in the proportion of fast-twitch and slow-twitch fibers, a change in the rate of calcium uptake by the sarcoplasmic reticulum (SR) could also contribute to the change. Accordingly, fragmented SR vesicles were prepared, but $^{45}Ca^{++}$ uptake was found to be unaffected by rat suspension. Consequently, it was concluded that soleus speeding did not result from a change in SR Ca^{++} uptake.

Part of the decline in muscle weight in spaceflight and the Morey-Holton method for rat suspension is caused by a decrease in muscle protein. This decline in protein could result from either decreased protein synthesis or increased protein degradation. Since degradation may involve lysosomal (acid) proteases, the activities of these proteases were measured and found to be elevated in suspended rats.

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SKELETAL MUSCLE METABOLISM IN HYPOKINETIC RATS

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Research focuses on the metabolic response of skeletal muscle to disuse. A broad range of metabolic pathways are being analyzed to determine which ones may be adversely affected with decreased muscular activity. The severity of these changes will be considered by evaluating the rapidity with which they return to normal when muscle use is restored.

In muscle, glutamine, together with alanine, serves as an important vehicle for transporting nitrogenous waste out of the tissue. Since muscle of growing animals in disuse shows growth failure and in some instances atrophy, excess nitrogen is available in these muscles. Hence, the role of glutamine takes on extra import under such conditions. In light of these ideas, it was rather surprising to find that the production of glutamine was slower in the disused soleus muscle. This slower production was characterized by a decreased ratio of tissue glutamine to glutamate due to a fall in tissue glutamine.

In muscle, a major source of nitrogen for the synthesis of alanine and glutamine are the branched chain amino acids, which transaminate with ketoglutarate to produce glutamate. One possible reason for the decreased synthesis of glutamine and glutamate might be decreased metabolism of the branched amino acids, which were present in the medium. In the soleus muscle, non-weight-bearing for 6 days increased the rate of flux through leucine aminotransferase. This effect could be accounted for by increased flux through ketoisocaproate dehydrogenase, the rate limiting step in the leucine degradative pathway. No differences were seen for the extensor digitorum longus muscles over the same period. This result is contrary to the data for decreased synthesis of glutamine. In fact, the data suggest that the soleus muscle could have a severe problem in removing nitrogen waste derived from the increased degradation of the branched-chain amino acids and protein.

Also considered, as part of the study of leucine metabolism, was the extent to which leucine was oxidized completely. This can be done by comparing the production of $^{14}CO_2$ from [1-^{14}C] and [U-^{14}C] leucine. If the ratio of production is only 0.17 then none of the leucine molecule is completely oxidized. Since both soleus and extensor digitorum longus muscles showed ratios significantly above this value leucine catabolism in soleus muscles of hypokinetic animals would increase the supply of acetyl CoA oxidized in the citric acid cycle.

The slower production of glutamine could have been due to several factors: (1) decreased concentrations of glutamate or ammonia, which are substrates for the glutamine synthetase reaction; (2) less ATP as an energy source for the reaction; or (3) a lower activity of glutamine synthetase. As noted above, tissue glutamate levels are not lowered so that this is not a limiting factor. Furthermore, the tissue concentration of ATP is elevated and not reduced in the soleus muscle in disuse.

In light of these findings it seemed likely that the activity of glutamine synthetase might be reduced. However, assays of this activity showed a 3-fold higher activity in the hypokinetic/hypodynamic soleus muscles. Despite a greater amount of enzyme, these muscles produced less glutamine. The remaining possibility as a defect in the ammonia availability. Determination of the dose-response curve for extracellular ammonium chloride during production of glutamine showed that indeed this must be true. At a high concentration of ammonium chloride (1mM) the hypokinetic soleus muscle produced considerably more glutamine. In fact the ratios of these apparent Vmax values were similar to the ratio of enzyme activities. Calculation of the Km values for extracellular ammonia showed a much higher value for the disused muscle. This result suggests that either ammonia transport is altered in hypokinesia or basal tissue ammonia concentration is lower, thus requiring more external ammonia to achieve the intracellular Km. In support of the latter idea, total adenine nucleotides are higher in hypokinesia, which is indicative of decreased ammonia production via the purine nucleotide cycle.

The effects of passive stretch in reducing the metabolic responses to disuse have also been studied by casting one limb of the hypokinetic rats in a dorsal flexed position to stretch the soleus muscle. Passive stretch prevented atrophy and growth failure of the muscle and actually caused a significant hypertrophy. This enlargement of the soleus muscle was borne out by more rapid rates of protein synthesis and slower rates of protein degradation in the stretched compared with the contralateral leg muscle. Passive stretch also prevented growth failure of the plantaris and gastrocnemius muscles. Preliminary results suggest that passive stretch may also reduce other metabolic responses such as the changes in the metabolism of ammonia and glutamine.

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EFFECTS OF SIMULATED WEIGHTLESSNESS ON MEIOSIS, FERTILIZATION, AND EARLY EMBRYONIC DEVELOPMENT IN MICE

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Research is directed toward elucidating the extent to which reproduction in mammals will (or will not) be affected during spaceflight conditions. The specific aspect being focused on is microgravity; the reproductive stages under study include meiosis, fertilization, and early embryonic cell division. These studies in turn will contribute to understanding of the environmental factors important for proper cell functioning during development and differentiation.

In order to experimentally simulate a microgravity environment, the initial objective was to develop a clinostat suitable for mammalian tissue culture; in particular, for culture of mouse oocytes, ova, and embryos. In 1983, this approach was first used to ask what effect rotating mouse oocytes in the clinostat would have on: (1) germinal vesicle breakdown, (2) progression to Metaphase I and Metaphase II of meiotic prophase, and (3) extrusion of the first polar body. Second, the original clinostat was modified to include a vertically rotating culture dish as a control for vibration, etc., in the experimental condition (horizontal rotation). Third, these studies are being extended to examine the effects of this altered gravity environment on in vitro fertilization in the mouse.

For the studies on the effects of clinostat rotation on meiosis, oocytes were recovered from ovarian follicles from CD-1 mice and placed immediately ($\leq$10 min.) into the tissue culture system. Incubation was conducted under clinostat rotation or control static conditions for 16 hr. The oocytes were then removed, examined under a dissecting microscope for polar body formation and gross morphological appearance, and then processed by cytogenetic techniques which permit a precise assessment of meiotic stage and chromosomal integrity. Previous experiments had shown that mouse oocytes cultured in the conditions adapted for use on a clinostat, but without rotation, yielded meiotic maturation indistinguishable from oocytes cultured under routine laboratory conditions.

A total of 59 experiments have been analyzed, representing rotations at 0 (control static), 1/4, 1, 10, 30, and 100 rpm. Qualitative evaluation of the condition of the cells immediately after culture on the clinostat revealed that there were no obviously damaged or fragmented cells, even at a rotation of 100

rpm. Cellular features such as the integrity of the zona pellucida, granularity of the cytoplasm, and clumping or necrosis of the granulosa cells were similar in the rotated vs. control oocytes. To date, there are no statistically significant differences in the percentage of germinal vesicle breakdown between static oocytes and oocytes cultured on the clinostat at any of the rotation speeds. This observation is consistent with what is known about meiotic maturation in other systems and has added to the understanding of which stages of meiotic maturation might be sensitive to changes in the environment (here, an altered gravitational field). That is, germinal vesicle breakdown is believed to be initiated within 30 min. of release from the ovarian follicle. Therefore, an effect would have to act very quickly to alter these events. In contrast, the period of chromosome alignment and movement is much more extended (~14-16 hrs.). These events are also highly dependent on cytoskeletal structure and relative orientation; thus they might be more susceptible to environmental changes.

In contrast, there is an indication of a slight difference in rate of progression through to Metaphase II among the rotated oocytes, particularly those rotated at 1 rpm. To determine if this trend is statistically significant, additional experiments are being completed. However, it was considered important to rule out the possibility that such differences could result from a feature of the clinostat culture system other than rotating the cells relative to their orientation in a gravitational field. Vibration could be such a feature. Therefore, a modification of the clinostat was developed this year. It consists of the addition of two bevel gears set at a perpendicular angle to each other, such that simultaneous horizontal and vertical rotations are achieved. This modified clinostat is now being used in all experiments.

Finally, experiments examining the effect of rotating oocytes on in vitro fertilization have just begun. As before, the first step has been to determine if the standard culture conditions can be modified for use on the clinostat. Fortunately, it was found that both the atmospheric conditions and the culture vessel used in the meiotic maturation experiments can be used in fertilization experiments. The major change lay in the selection of a different culture medium, a modified Whittens medium adapted from Hoppe and Pitts. For these experiments, oocytes are recovered from the ampulae of B6D2F1 mice (F1 of C57Bl/6J x DBA/2J) which had been superovulated. The oocytes, surrounded by their cumulus mass, are placed with capacitated sperm at a concentration of ~4×10^4 sperm/ml and rotated for 4 hr. The cultures are then removed from the clinostat, transferrred to fresh culture dishes, and rotated for an additional 4-8 hr. The embryos are fixed with glutaraldehyde and then formalin, stained with toluidine blue, and examined for the following endpoints: (1) extrusion of the second polar body; (2) formation of both a

male and female pronucleus; and (3) percentage of artificially activated eggs. Based on observations to date on meiotic maturation, it might be predicted that later rather than earlier events in fertilization would be more likely affected, since they would be exposed to longer rotation times.

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SPECIAL ACTIVITIES

RESEARCH ASSOCIATE PROGRAM IN SPACE BIOLOGY

The NASA Shuttle Program is currently providing a unique opportunity to conduct biological research in outer space and to continue relevant ground-based Space Biology research. To maximize the potential for Space Biology as an emerging discipline, there is a need to develop a cadre of scientists who are interested in working in Space Biology. The program utilizes a competitive grant process and awards are made to the more promising applicants. It is anticipated that these scientists will develop their research careers in the newly evolving discipline of Space Biology.

The grant is designed to make awards on January 1 and July 1 of each year, following semiannual announcements. An Award Committee reviews the proposals and recommends appointments. The awards are for a 1-yr. period with the possibility of renewal for a second year. The program began on June 1, 1980, and since then 32 awards have been made. The recipients have been 20 scientists, including zoologists, developmental biologists, botanists, and physiologists (animal and plant). Twelve of these individuals have received a second year of funding. These scientists work in NASA-funded laboratories or laboratories that can provide the necessary facilities and environment for specialized Space Biology projects. Originally (June 1980) there were 19 laboratories participating. Presently (April 1984), there are 46 laboratories participating.

In addition to the salary stipend, the awardees are encouraged to attend and present papers at two national meetings: 1) the annual AIBS/NASA meeting, and 2) a national society meeting of their choice.

The success of the program is readily measured in terms of the quality and quantity of publications as well as the job opportunities and placements of the Research Associates. Individuals have obtained positions in college or university settings and in research laboratories. A large number of publications have resulted from this program.

The grant is administered through the University of Louisville, Louisville, Kentucky. Dr. X.J. Musacchia, Dean of the Graduate School, is the Project Director and Science Advisor.

Meetings

Organized or participation by the Space Biology Program:

- "Space Biology Workshop," DFVLR-Institute of Aerospace Medicine, Cologne, Germany, March 9-11, 1983
- "SL-2 Plant Experiment Meeting," AIBS, Rosslyn, Virginia, USA, March 21, 1983
- "Primate Workshop,", FASEB, Bethesda, Maryland, USA, April 17-19, 1983
- "Spaceflight Centrifuge Workshop," FASEB, Bethesda, Maryland, USA, April 26, 1983
- "Thirteenth Intersociety Conference on Environmental Systems," San Francisco, California, USA, July 11-13, 1983
- "Fifth Annual Meeting of the IUPS Commission on Gravitational Physiology," Moscow, USSR, July 26-29, 1983
- "Fifty-ninth Annual Meeting of the American Society for Plant Physiologists," Colorado State University, Fort Collins, USA, August 7-11, 1983
- "Symposium on Gravitational Physiology," Syndey, Australia, August 28-September 3, 1983
- "Annual Symposium of the NASA Space Biology Program," Rosslyn, Virginia, USA, October 12-14, 1983
- "Space Plant Growth Unit Workshop," Rosslyn, Virginia, USA, February 8, 1984.

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16. Abstract

Summaries of NASA's Space Biology Program projects. The goals, objectives, accomplishments, and future plans of each project are described in this publication as individual technical summaries. Also includes bibliographies for each project.

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